Express Mail No.: ED 663 710 285 US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appl. No.	:	10/004,494	Confirmation No. 9399
Applicant	:	Chang, Yung-Fu	
Filed	:	November 2, 2001	
Title	:	*Ehrlichia canis* genes and vaccines	
Art Unit	:	1632	
Examiner	:	Woitach, Joseph T.	
Docket No.	:	CRF-2322 CIP (1258-006 CIP)	
Customer No.	:	20874	

DECLARATION OF YUNG-FU CHANG, Ph.D.
UNDER 37 C.F.R. § 1.132

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, YUNG-FU CHANG, Ph.D. do declare that:

1. I am the inventor of the invention disclosed and claimed in the above-identified patent application. A copy of my curriculum vitae is attached as Exhibit 1.

2. I am a Professor of Population Medicine & Diagnostic Sciences at the College of Veterinary Medicine of Cornell University. The Cornell Research Foundation, Inc., a not-for-profit affiliated corporation of Cornell University, is the assignee of the above-identified patent application.

3. I have read and understood the above-identified patent application and the Office Action dated January 24, 2005 ("Office Action") issued in connection therewith.

4. I understand that the above-identified application is a continuation-in-part application of prior application Serial No. 09/358,322, filed July 21, 1999 (now abandoned). I understand that the claimed priority dates are July 21, 1999 and November 2, 2001.

5. I understand that in the Office Action, the Examiner rejected pending claims 1, 2, 5-11, 26-31 and 47-50 (Exhibit 2) under 35 U.S.C. 112, first paragraph, as allegedly not enabled by the specification as filed. The Examiner particularly contends that the specification does not enable an artisan to make and use the *Ehrlichia canis* genes and vaccines of the invention because:

(a) "the art of gene therapy, in particular the delivery of a DNA vaccine which encode[s] undefined immunogenic epitopes, is highly unpredictable as recognized in the prior art," and

(b) "the specification as filed does not provide sufficient guidance, evidence and exemplification as to how an artisan would have carried out the claimed methods for expression of any of the specific cloned genes from *E. canis*, wherein the encoded protein stimulates the appropriate immune response such that a prophylactic effect is achieved in said subject." Office Action, page 6.

6. I am making this declaration to explain that as of the earliest claimed priority date of the above-identified application, a skilled artisan could have used the teachings of the specification, coupled with routine methods and knowledge in the art, to practice the claimed

invention recited in claims 1, 2, 5-11, 26-31 and 47-50 without undue experimentation. Furthermore, as of the earliest claimed priority date, a skilled artisan would have been able to use the methods set forth in the specification to routinely determine which of the five disclosed open reading frames (ORFs), as recited in claim 1 sections (a)-(e) are detectable by antisera used in the isolation of the genomic fragment, and therefore, used to practice the invention.

7. Pending claims 1, 2, 5-11, 26-31 and 47-50 (Exhibit 2) are drawn to certain embodiments of the invention, specifically: a recombinant DNA (claim 1, claim 2 depending therefrom, claim 47, claim 49 depending therefrom); a vaccine that protects dogs against *E. canis* infection (claim 5); a vaccine comprising a vector capable of expressing a recombinant DNA inserted into said vector (claim 6, claims 7-11 depending therefrom); a method of creating a vaccine against *Ehrlichia canis* (claim 26, claims 27-31 depending therefrom); and a vector capable of expressing a recombinant DNA (claim 48, claim 50). As detailed below, as of the earliest claimed priority date of the application, a skilled artisan, armed with the teachings of the specification and using routine experimentation and screening methods known in the art, could have determined, without undue experimentation, which protein epitope or which of the five proposed open reading frames encodes a protein that reacts with the antisera used in the isolation of the genomic fragment. A skilled artisan could also have obtained, without undue experimentation, useful protein epitopes or immunoreactive proteins with which to practice the claimed invention.

8. It should be noted that using methods disclosed in the instant specification and routine methods known in the art, Teng et al. (2003a, Cloning and characterization of an

Ehrlichia canis gene encoding a protein localized to the morula membrane, Infect. Immun. 71(4): 2218-2225; Exhibit 3) determined that one of the ORFs disclosed in the instant specification, mmpA (erlichial morula membrane protein A) can be used for the production of a putative *E. canis* vaccine. Specifically, the gene encoding MmpA was cloned by screening an *Ehrlichia canis* expression library with convalescent dog sera, and isolating three positive clones. Sequence analysis of the insert DNAs from all three clones indicated an open reading frame with a size of 666 bp that encodes MmpA. The structural analysis of MmpA indicated that it is a transmembrane protein with extreme hydrophobicity, making it a good candidate for a protein that possibly mediates bacterium-host cell interaction during *E. canis* infection. Southern blot analysis of the HindIII-digested chromosomal DNA demonstrated the presence of a single copy of the MmpA gene in *E. canis* and *Ehrlichia chaffeensis* but not in the human granulocytic ehrlichiosis agent. Using routine methods in the art, the mmpA gene was amplified, cloned, and expressed as a fusion protein. Polyclonal antibodies to the recombinant protein (rMmpA) were raised in rabbits. Western blot analysis of *E. canis* and *E. chaffeensis* lysates with the anti-rMmpA serum resulted in the presence of an MmpA band only in *E. canis*, not in *E. chaffeenesis*. Sera from dogs which were either naturally or experimentally infected with *E. canis* recognized the recombinant protein. Double immunofluorescence confocal microscopy studies demonstrated that MmpA was localized mainly on the morula membrane of *E. canis*. Since the morula membrane is the interface between the ehrlichial growing environment and the host cytoplasm, it was concluded that MmpA plays a role in bacterium-host cell interactions during *E. canis* infection and can therefore be used for the production of a vaccine.

9. Furthermore, it should be noted that using methods disclosed in the instant specification and routine methods known in the art as of the earliest claimed priority date, Teng et al. (2003b, Cloning and characterization of putative zinc protease genes of *Ehrlichia canis*, DNA Sequence 14(2): 109-121; Exhibit 4) determined that two additional ORFs disclosed in the instant specification, ProA and ProB, code for proteins that are expressed in *E. canis* and that can be used in making an *E. canis* vaccine. Specifically, ProA and ProB were determined by routine methods to be putative zinc protease genes encoded by a putative zinc protease gene operon. Routine BLAST analyses determined that ProA and ProB share 20-30% identities with members of the eukaryotic mitochondrial processing peptidase (MPP) subfamily, which are heterodimers containing alpha and beta subunits. The subunits share 20% of identity, but only MPP-beta contains a conserved zinc-binding motif, His-Xaa-Xaa-Glu-His (HXXEH). proA and proB were also found to be detectable in *E. canis* and *Ehrlichia chaffeensis*, but not in *Anaplasma phagocytophila*. 5'-RACE revealed that the 5' end of the proA mRNA is heterogeneous, containing additional adenine residues that may be directed by pseudo-templated transcription. Although ProA was identified in both *E. canis* and *E. chaffeensis*, ProB was detected only in *E. canis*. ProA and ProB were both detectable in *E. canis*-infected DH82 cells, demonstrating that the ORFs are transcribed and translated into ProA and ProB proteins. Sera from dogs, which were either naturally or experimentally infected with *E. canis*, recognized both the recombinant protein antigens.

10. Furthermore, as of the earliest claimed priority date, it would have been routine for a skilled artisan, armed with the methods disclosed in the instant specification and routine methods known in the art, to determined that proteins encoded by the proposed open reading frames produce a prophylactic immune response to *E. canis* infection as encompassed by

claims 1, 2, 5-11 and 26-31 and 47-50. Using the methods disclosed in the instant specification and routine methods known in the art as of the earliest claimed priority date, Teng et al. 2003a (Exhibit 3) and Teng et al. 2003b (Exhibit 4) determined which proteins (or parts of proteins) contain immunogenic epitopes.

Double immunofluorescence confocal microscopy studies conducted by Teng et al. 2003a demonstrated that MmpA was localized mainly on the morula membrane of *E. canis*, interface between the ehrlichial growing environment and the host cytoplasm.

Studies conducted by Teng et al. 2003b provide further confirmation of the example disclosed at page 11, line 27 to page 12, line 2 of the specification, which shows that ProA and ProB can serve as target antigens for a DNA vaccine. Teng et al. 2003b showed that *E. canis* expresses ProA and ProB in dogs that are naturally infected through tick bites or artificially infected by IV inoculation of E. canis-infected DH82 cells, since sera from experimentally or naturally E. canis-infected dogs recognized both r (recombinant) ProA and rProB. In addition, dog anti-E.canis serum detected ProA and ProB in E. canis-infected DH82 cells in Western blot and indirect immunofluorescent assays. These results demonstrate clear connections between the MmpA, ProA and ProB ORFs and protein epitopes that are recognized by the antisera from dogs infected with *E. canis*. The results also demonstrate that the encoded proteins from these ORFs could serve as potential vaccines for *E. canis* infection. The antigenicity and general expression of the MmpA, ProA and ProB proteins also demonstrate that these three proteins can serve as good tools for serodiagnosis of *E. canis* infection.

11. It should also be noted that methods disclosed in the instant specification, along with routine methods known in the art, have been used to demonstrate in other species (e.g.,

horses) a clear connection between a potential open reading frame and a protein epitope that is recognized by an antisera from an infected subject (Palaniappan et al., 2002, Cloning and molecular characterization of an immunogenic LigA protein of *Leptospira interrogans*, Infect. Immun. 70(11): 5924-5930; Exhibit 5). Palaniappan et al. isolated a clone expressing a novel immunoreactive leptospiral immunoglobulin-like protein A of 130 kDa (LigA) from *Leptospira interrogans* serovar pomona type kennewicki by routine screening a genomic DNA library with serum from an infected mare. LigA was demonstrated to be encoded by an open reading frame of 3,675 bp, and the deduced amino acid sequence consists of a series of 90-amino-acid tandem repeats. A search of the NCBI database found that homology of the LigA repeat region was limited to an immunoglobulin-like domain of the bacterial intimin binding protein of Escherichia coli, the cell adhesion domain of *Clostridium acetobutylicum*, and the invasin of *Yersinia pestis*. Secondary structure prediction analysis indicated that LigA consists mostly of beta sheets with a few alpha-helical regions. No LigA was detectable by immunoblot analysis of lysates of the leptospires grown in vitro at 30°C or when cultures were shifted to 37°C. Immunohistochemistry on kidney from leptospira-infected hamsters, however, demonstrated LigA expression. These findings suggest that LigA is specifically induced only in vivo. Sera from infected horses strongly recognize LigA. LigA is the first leptospiral protein described to have 12 tandem repeats and is also the first to be expressed only during infection. Thus, it was concluded that LigA has value as a protective immunogen in vaccines and in serodiagnosis.

12. Furthermore, it would have been routine for a skilled artisan, using methods well known in the art, to produce the effects of immunization through the methods recited in the pending claims. As of the earliest claimed priority date, it was routine and well-known in

the art how to produce a vaccine derived from a recombinant protein and to use it to produce a prophylactic immune response in a subject administered the vaccine. For example, four years before the earliest claimed priority date, Chang et al. published a report (1995, Recombinant OspA protects dogs against infection and disease caused by *Borrelia burgdorferi*, Infect. Immun. 63(9): 3543-3549; Exhibit 6, Ref. AM of record) vaccinated dogs with recombinant OspA (derived from *Borrelia burgdorferi* B31, the spirochete that causes Lyme disease) alone or with adjuvant. Six weeks or 6 months after the first vaccination, vaccinated and nonvaccinated dogs were challenged by exposure to adult ticks (*Ixodes scapularis*) naturally that were infected with the Lyme disease spirochete *B. burgdorferi* (tick infection rate, >60%). Protection from infection was evaluated by culture for *B. burgdorferi* from skin biopsies taken near the sites of tick bites. *B. burgdorferi* was not isolated from any of the vaccinated dogs. In contrast, 12 of 13 control dogs challenged by exposure to the ticks were culture positive and 8 of 13 control dogs showed signs of arthritis in joints, while only 1 of the 22 vaccinated dogs had a single focus of mild inflammation in a single joint. At the time of the challenge, the vaccinated dogs had antibody to *B. burgdorferi*, which was demonstrable by kinetic enzyme-linked immunosorbent assay, Western blotting (immunoblotting), and a serum growth inhibition assay. The vaccinal antibody declined gradually after the challenge, especially in dogs vaccinated with OspA without adjuvants. Antibodies in the challenge control dogs were only detectable by 4 to 6 weeks after the challenge and remained at high levels until the termination of the study. By routine Western blot analysis, antibodies to OspA were observed to first appear in the sera of vaccinated dogs 3 weeks after the first vaccination. The absence of additional bands after the challenge suggests that infection in vaccinated dogs was blocked. The results of this study show that

vaccination with recombinant OspA protected dogs against infection and disease after an experimental challenge with *B. burgdorferi* by exposure to ticks.

13. In addition, it should be noted that as of the earliest claimed priority date, it would have required only routine experimentation for a skilled artisan, using the methods disclosed at pages 12-14 of the specification, to identify T-cell epitopes for use in an epitope vaccine. As disclosed at pages 12-14 of the specification, mapping of T-cell epitopes was well known in the art as of the earliest claimed priority date. Equipped with such routine knowledge, a skilled artisan would have been able to synthesize short, overlapping peptide sequences for a protein encoded by a proposed ORF of the instant invention, and to test these putative epitopes, in dogs immunized with the protein, for T cell stimulatory and IFN-gamma (γ) inducing properties. I also emphasize that it would have been routine and required no undue experimentation, as of the earliest claimed priority date, for a skilled artisan to determine which of several vectors or promoters routinely used in the art (several examples of which are disclosed at page 12 of the specification) to choose from for use in induction of an immune response.

14. I also emphasize that although some experimentation would have been required to deliver a polynucleotide via a DNA vaccine for the production of a protein epitope, such experimentation would not have been undue. I therefore disagree with the Examiner's assessment, at page 11 of the Office Action, that a skilled artisan would require examples of how the recited and claimed methods of producing a DNA vaccine differ from those presently found in the art in order to make and use the invention as claimed. Such examples

are not needed since the practice of the claimed methods would require only routine experimentation using methods well known in the art.

15. Taken together, the teachings of Exhibits 3-6, as described above, rebut the rejection at pages 5-12 of the Office Action that the claimed invention is not enabled because one skilled in the art would have been required to perform undue experimentation to practice the invention. As discussed hereinabove, the methodology and guidance disclosed in the instant specification, coupled with routine knowledge in the field, would have enabled a skilled artisan to determine, as of the earliest claimed priority date and without undue experimentation (1) which protein epitope or ORF encodes a protein that reacts with antisera from an infected dog, (2) which of the proposed ORFs encodes an *E. canis* protein that produces a prophylactic immune response to *E. canis* infection, and (3) which of the methods disclosed in the specification can be used for expression of a proposed ORF that results in a prophylactic immune response.

16. In sum, the analysis presented above indicates that the approach disclosed in the specification, coupled with knowledge well-known in the art of vaccine production, would have enabled a skilled artisan to make and use the invention as of the earliest claimed priority date. When a skilled artisan in the field is given extensive guidance to appropriate protocols throughout the instant specification, referenced by citations to specific literature and incorporating many techniques that are extremely well-known and routine in the art, any experimentation that may be necessary becomes routine. The skilled artisan knows that the methodologies disclosed in the instant specification *will* work, and indeed, the above-described references demonstrate that the disclosed methodologies *do* work.

17. I declare further that all statements made in this Declaration of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 9-22-05

YUNG-FU CHANG, Ph.D.

Attachments:

Exhibit 1: Curriculum vitae of YUNG-FU CHANG, Ph.D.

Exhibit 2: Pending claims 1, 2, 5-11 and 26-31 and 47-50

Exhibit 3: Teng et al., 2003a, Cloning and characterization of an *Ehrlichia canis* gene encoding a protein localized to the morula membrane, Infect. Immun. 71(4): 2218-2225

Exhibit 4: Teng et al. (2003b, Cloning and characterization of putative zinc protease genes of *Ehrlichia canis*, DNA Sequence 14(2): 109-121

Exhibit 5: Palaniappan et al., 2002, Cloning and molecular characterization of an immunogenic LigA protein of *Leptospira interrogans*, Infect. Immun. 70(11): 5924-5930

Exhibit 6: Chang et al., 1995, Recombinant OspA protects dogs against infection and disease caused by *Borrelia burgdorferi*, Infect. Immun. 63(9): 3543-3549; Ref. AM of record

Exhibit 1

Principal Investigator/Program Director (Last, first, middle): Yung-Fu Chang

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
Yung-Fu Chang	Professor

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE	YEAR	FIELD OF STUDY
National Pintung University of Science and Technology	DVM	1974	Veterinary Medicine
University of Idaho	MS	1981	Immunology
Texas A&M University, College of Veterinary Medicine	Ph.D.	1984	Microbiology
Texas A&M University, College of Medicine	Post-doc	1989	Molecular Biology

RESEARCH AND PROFESSIONAL EXPERIENCE:

1974-1979 Assistant Pathologist, Veterinary Pathology Division, Taiwan Provincial Research Institute for Animal Health, Taiwan

1984-1985 Research Associate, Medical Biochemistry and Genetics, College of Medicine, Texas A&M University, College Station, Texas

1986-1989 Assistant Research Scientist, Medical Biochemistry and Genetics, College of Medicine, Texas A&M University, College Station, Texas

1989-present Assistant/Associate/Full Professor, Department of Population Medicine & Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, New York.

2003(Jan. to July). Visiting Professor, Department of Infectious disease and Medicine, College of Medicine, Stanford University. Stanford, California.

PROFESSIONAL SOCIETIES: Society of Sigma Xi, American Society for Microbiology, The Society of PHi Zeta, American Association for the Advancement of Science.

PATENTS:

Leukotoxin gene from *Pasteurella suis*: US Patent: 5,559,008

DNA encoding *Actinobacillus pleuropneuminae* hemolysin. US Patent: 5,641,653.

Recombinant vaccine for porcine pleuropnuemonia US Patent:5,803,190

Ehrlichia canis gene and vaccine. Australian Patent No. 772946.

Helicobacter bizzozeronii urease and its use in diagnostic and treatment methods (Pending).

Helicobacter bizzozeronii outer membrane protein and its use in diagnostic and treatment methods (Pending).

Leptospira interrogans serovar Pomona adhesin gene and vaccine (Pending).

EMCHIPS-Enhanced Microarray Construction and Hybridization Improvement using a polymer Surface. (Disclosure)

RESEARCH INTERESTS: Molecular biology of infectious disease; DNA and recombinant subunit vaccine development; molecular basis of bacterial pathogenesis; immunopathology of

infectious disease; DNA probes development; host defense mechanisms; Microarray for diagnosis & pathogenesis.

Selected peer-reviewed publications (in chronological order): (from 2000- present)

Chang, Y. F., V. Novosel, S. P. McDonough, C. F. Chang, R. H. Jacobson, T. Divers, F. W. Quimby, S. Shin, and D. H. Lein. 2000. Experimental infection of ponies with *Borrelia burgdorferi* by exposure to Ixodid ticks. Vet. Pathol. 37: 68-76.

Chang, Y. F., S. P. McDonough, K.S. Shin, C. F. Chang, and T. Divers. 2000. Human granulocytic ehrlichiosis agent (HGE) infection in a pony vaccinated with recombinant OspA vaccine and challenged by exposure to naturally infected ticks. Clin. Diag. Lab. Immunol. 7: 68-71.

Valentine, B. A., S. P. McDonough, **Y. F. Chang**, and A. J. Vonderchek. 2000. Polysaccharide storage myopathy in non-quarter horse, draft, or warmblood related horses and ponies. Vet. Pathol. 37: 193-196.

Simpson, K.W., D. Strauss-Ayali, E. Scanziani, R. K. Straubinger, P. L. McDonough, A.F. Straubinger, **Y. F. Chang**, C. Domeneghini, N. Arebi, and J. Calm. 2000. *Helicobacter felis* infection is associated with lymphpoid follicular hyperplasia and mild gastritis but normal gastric secretory function in cats. Infect. Immun. 68: 779-790.

Divers, T. J., **Y. F. Chang**, R. H. Jacobson, and S. P. McDonough. 2001. Lyme disease in horses. Comp. Contin. Ed. Pract. Vet. 23: 375-381.

Simpson, K.W., D. Strauss-Ayali, E. Scanziani, R. K. Straubinger, P. L. McDonough, A. F. Straubinger, **Y. F. Chang**, M. Esteves, J. G. Fox, C. Domeneghini, N. Arebi, and J. Calam. 2001. Gastric secretory function in cats with *Helicobacter pylori* infection. Helicobacter 6: 1-14.

Chang, Y. F., V. Novosel, C. F. Chang, B. A. Summers, D. P. Ma, Y. W. Chiang, W. M. Acree, H. J. Chu, S. Shin, and D. H. Lein. 2001. Experimental induction of chronic borreliosis in adult dogs exposed *to Borrelia burgdorferi*-infected ticks and treated with dexamethasone. Am. J. Vet. Res. 62: 1104-1112.

Tan, H., R. G. Creech, J. N. Jenkins, **Y. F. Chang**, and D. P. Ma. 2002. Cloning and expression analysis of two cotton (*Gossypium hirsutm* L.) genes encoding cell wall proline-rich proteins. DNA. Seq. 12: 367-380.

Chang, C.-F., T.-M.Yeh, C.C. Chou, **Y.F. Chang**, T.S. Chiang. 2002. Antimicrobial susceptibility and plasmid analysis of *Actinobacillus pleuropneumoniae* isolated in Taiwan. Vet. Microbiol. 84:169-177.

Chang, C. F., L. C. Chang, **Y. F. Chang**, M. Chen, and T. S. Chiang. 2002. Antimicrobial susceptible of *Actinobacillus pleuropneumoniae*, *Escherichia coli*, and *Salmonella choleraesuis* recovered from Taiwanese swine. J. Vet. Diagn. Invest. 14:153-157.

Raghavan, P. U. M., **Y. F. Chang**, S. S. D. Jusuf, S. Artiushin, J. F. Timoney, S. P. McDonough, S. C. Barr, T. J. Divers, P. McDonough, K. W. Simpson, and H. Mohammed. 2002. Cloning and molecular characterization of an immunogenic LigA of *Leptospira interrogans*. Infect. Immun. 70:5924-5930.

Chang, C.F., W.H.Lin, T.M. Yeh, T.S. Chiang, and **Y. F. Chang**. 2002. Antimicrobial susceptibility of *Riemerella anatipestifer* isolated from ducks and the efficacy of ceftiofur treatment. J. Vet. Diagn. Invest. 15:26-29.

Dheenadhayalan, V., K. S. Shin, C. F. Chang, C. D. Chang, S. J. Wang, S. P. McDonough, P. L. McDonough, S. Shin, A. Torres, and **Y. F. Chang**. 2002. Cloning and characterization of the genes coding for antigen 85A, 85B and 85C of *Mycobacterium*

avium subsp. *paratuberculosis.* DNA Seq. 13:287-294.

Zhu, J., C. H. Teng, C. F. Chang, C. D. Chang, K. W. Simpson, C. Wei, P. McDonough, S. McDonough, and **Y. F. Chang**. 2002. Cloning and characterization of a *Helicobacter bizzozeronii* urease gene cluster. DNA. Seq. 13:321-331.

Wu, W. S., P. C. Hsieh, T. M. Huang, **Y. F. Chang**, and C. F. Chang. 2002. Cloning and characterization of an iron regulated locus, *iroA*, in *Salmonella entrica* serovar Choleraesuis. DNA. Seq. 13:333-341.

Zhu, J., Y.M. Hsu, K. W. Simpson, H. Tu, C. F. Chang, and **Y. F. Chang**. 2003. Cloning and characterization of a *Helicobacter bizzozeronii* Lip18 gene . DNA. Seq. 14:87-94.

Teng, C. H., and **Y. F. Chang**. 2003. Cloning and characterization of an *Ehrlichia canis* encoding a protein localized to the morula membrane. Infect. Immun. 71:2218-25.

Teng, C. H., and **Y. F. Chang**. 2003. Cloning and characterization of putative zinc protease genes of *Ehrlichia canis*. DNA seq. 14:109-121.

Hsu, Y. M., N. Chin, C. F. Chang, and **Y. F. Chang**. 2003. Cloning and characterization of *Actinobacillus pleuropneumoniae fur* gene and its role in regulation of ApxI and AfuABC expression. DNA seq. 14:169-181.

Santos, L. R. D., S. M. Barrouin-Melo, **Y. F. Chang**, J. Olson, S. P. McDonough, F. Quimby, W. L. C. D. Santos, L. C. Pontes-de-Carvalho, G. G. Oliveria. 2004. Recombinant single-chain interleukin-12 induces interferon mRNA expression in peripheral blood mononuclear cells of dogs with visceral leishmaniasis. Vet. Immun. Immunopathol. 98:43-48.

Shin, S.J., **Y. F. Chang**, C. Huang, J. Zhu, L. Huang, H.S. Yoo, K.S. Shin, S. Stehman, S. J. Shin, and A. Torres. 2004. Development of a polymerase chain reaction test to confirm *Mycobacterium avium* subsp. paratuberculosis in cultures. J. Vet. Diagn. Invest. 16:116-120.

Huang, T.-M, **Y.-F. Chang**, and C.-F. Chang. 2004. Detection of Mutations in the *gyrA* Gene and Class I Integron from Quinolone-Resistant *Salmonella enterica* serovar Choleraesuis isolates in Taiwan. Vet. Microbiol. 100:247-254.

Huang, T.-M, **Y.-F. Chang**, and C.-F. Chang. 2004. Antimocribial susceptibility and resistance gene determinants in clinical *Escherichia coli* and *Salmonella enterica* serovar Choleraesuis swine isolates. Taiwan Vet. J. 30:116-124.

Shin, S.J., H.S. Yoo, S. P. McDonough, and **Y. F. Chang**. 2004. Comparative antibody response of five recombinant antigens in related to bacterial shedding levels and development of serological diagnosis based on 35 kDa antigen for *M. avium* subsp. paratuberculosis. J. Vet. Sci. 5:111-117.

Palaniappan, R. U., **Y. F. Chang**, F. Hassan, S.P. McDonough, M. Pough, S.C. Barr, K. W. Simpson, H. O. Mohammed, S. Shin, P. McDonough, R. Zuerner, J. Qu, & B. Roe. 2004. Expression of leptospiral immunoglobulin-like protein from *Leptospiral interrogans* and evaluation of its diagnostic potential in kinetic enzyme linked immunosorbent assay. J. Med. Microbiol. 53: 975-84.

Wang, Z., Z. Yuan, M. Matsumoto, U. R. Hengge and **Y. F. Chang**. 2005. Immune responses with DNA vaccines encoded different gene fragments of severe acute respiratory syndrome coronavirus in BALB/c mice. BioChem. BioPhy. Res. Com. 327:130-135.

Palaniappan, R. U., **Y. F. Chang**, C. F. Chang, M. J. Pan, C. W. Yang, P. Harpending, S. P. McDonough, E. Dubovi, J. Qu, B. Roe and T. Divers. 2005. Evaluation of *lig*-based conventional and real time PCR for the detection of pathogenic leptospires. Mol. and Cell. Probes. 19:111-117.

Wiinberg, B., A. Spohr, H. H. Dietz, T. Egelung, A. Greiter-Wilke, S. P. McDonough, J.

Olsen, S. Priestnall, **Y. F. Chang**, and K. W. Simpson. 2005. Quantitative analysis of inflamamtory and immune responses in dogs with gastritis and their relationship to *Helicobacter* ssp. infection. J. Vet. Intern. Med. 19:4-14.

Hsieh, W.J., **Y.F. Chang,** C. S. Chen, and M. J. Pan. 2005. Omp52 is a growth-phase-regulated outer membrane protein of *Leptospira santarosai* serovar Shermani. FEMS Microbiol. Let. 243:339-345.

Chang, Y.F., Y.W. Ku, C. F. Chang, C. D. Chang, S. P. McDonough, T. Divers, M. Pough, A. Torres. 2005. Antibiotic treatment of experimentally *Borrelia burgforferi*-infected ponies. Vet. Microbiol. In press.

Summers, B.A., R. K. Straubinger, A. F. Straubinger, R. H. Jacobson, **Y. F. Chang**, M. J.G. Appel. 2005. Histopathologic studies of experimental Lyme disease in the dog. J. Comp. Med. 133:1-13.

Shin, S.J., C.F. Chang, C. C. Chang, S. P. McDonough, B. Thompson, H.S. Yoo, and **Y. F. Chang**. 2005. In vitro cellular immune response to recombinant antigens of *Mycobacterium avium* subsp. Paratuberculosis. Infect. Immun. Accepted.

<u>Current Grant Support:</u>

Chang (PI-5%) 7/01/04-06/30/05, CAT: " Development of a serological test using recombinant antigens for animal leptospirosis".

Chang (PI-20%). 11/1/03-10/31/07, BRDC: Paratuberculosis: Novel DNA vaccine with single chain bovine IL-12 adjuvant.

Chang (PI-20%). 11/1/03-10/31/07, BRDC: Identification of *L. borgpetersenii* serovar Harjo virulence factors andvaccine development.
Chang (PI-10%). 4/1/03-31/07. NIH (N01-AI-30054; ZC002-03) Molecular Diagnosis (Microarray) of bacterial pathogens.

Chang (PI-5%). 10/1/04-9/30/07. CUAES Hatch project. Biosensor for rapid detection of *Mycobacterium avium* subsp. paratuberculosis.

Chang (PI-5%). 1/1/05-12-30/05. Cornell Collaborative program. Identification of virulence genes responsible for survival of *M. avium* subsp. paratuberculosis in bovine macrophages.

Chang (PI-5%). 11/1/04-10/30/05. USDA-APHIS Johnes' Disease: recombinant/DNA vaccine development.

Chang (PI-5%). 1/1/05-12-30/06. Zweig. Virulence factors of serovar Pomona and vaccine development.

Exhibit 2

Pending Claims - U.S. Patent Application 10/004,494

1 (original). A recombinant DNA comprising said DNA selected from the group consisting of:

a) a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 3;

b) a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 5;

c) a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 7;

d) a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 9;

e) a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 11; and

f) any portion of said DNA above that encodes a protein that elicits an immune response against *E. canis*.

2 (original). The recombinant DNA of claim 1 wherein said DNA encodes at least one immunogenic epitope.

3-4 (canceled).

5 (original). A vaccine wherein said vaccine protects dogs against *E. canis* infection.

6 (original). A vaccine comprising:

a) a vector capable of expressing a recombinant DNA inserted into said vector such that a recombinant protein is expressed when said vector is provided in an appropriate host; and

b) the recombinant DNA inserted into said vector wherein said DNA is selected from the group consisting of:

i) a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 3;

ii)a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 5;

iii)a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 7;

iv)a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 9;

v)a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 11; and

vi)any portion of said DNA above that encodes a protein fragment that is greater than 25 amino acids.

7 (original). The vaccine of claim 6, wherein said DNA further comprises DNA that encodes CpG motifs.

8 (original). The vaccine of claim 6 wherein said DNA further comprises a promoter selected from the group consisting of:

a) a cytomegalovirus (CMV) immediate early promoter;

b) a human tissue plasminogen activator gene (t-PA); and

c) promoter/enhancer region of a human elongation factor alpha (EF-1 α).

9 (original). The vaccine of claim 6, wherein said vector is selected from the group consisting of:

a) pcDNA3;

b) pC1;

c) VR1012; and

d) VR1020.

10 (original). The vaccine of claim 6 wherein said vaccine is administered into said host by a method selected from the group consisting of:

a) intramuscular injection;

b) intravenous injection; and

c) gene gun injection.

11 (original). The vaccine of claim 10, wherein said host is a dog.

12- 25 (canceled).

26 (original). A method of creating a vaccine against *Ehrlichia canis* comprising:

a) selecting a vector capable of expressing a recombinant DNA inserted into said vector; and

b) inserting a recombinant DNA into said vector such that a recombinant protein is expressed when said vector is provided in an appropriate host wherein said DNA is selected from the group consisting of:

i) a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 3;

ii) a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 5;

iii) a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 7;

iv) a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 9;

v) a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 11; and

vi) any portion of said DNA above that encodes a protein fragment that is greater than 25 amino acids.

27 (original). The method of claim 26, wherein said DNA further comprises DNA that encodes CpG motifs.

28 (original). The method of claim 26 wherein said DNA further comprises a promoter selected from the group consisting of:

a) a cytomegalovirus (CMV) immediate early promoter;

b) a human tissue plasminogen activator gene (t-PA); and

c) a promoter/enhancer region of a human elongation factor alpha (EF-1 α).

29 (original). The method of claim 26, wherein said vector is selected from the group consisting of:

a) pcDNA3;

b) pC1;

c) VR1012; and

d) VR1020.

30 (original). The method of claim 26 wherein said vaccine is injected into said host in a manner selected from the group consisting of:

a) intramuscular injection;

b) intravenous injection; and

c) gene gun injection.

31 (original). The method of claim 30, wherein said host is a dog.

32-46 (canceled).

47 (original). A recombinant DNA comprising said DNA selected from the group consisting of

a) a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 3;

b) a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 5;

c) a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ.ID. NO. 7;

d) a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 9; and

e) a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 11.

48 (original). A vector capable of expressing a recombinant DNA comprising:

a) a recombinant DNA inserted into said vector such that a recombinant protein is expressed when said vector is provided in an appropriate host wherein said DNA is selected from the group consisting of:

i) a recombinant DNA sequence that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 3;

ii) a recombinant DNA sequence that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 5;

iii) a recombinant DNA sequence that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 7;

iv) a recombinant DNA sequence that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 9;

v) a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 11; and

vi) any portion of said DNA above that encodes a protein that elicits an immune response against *E. canis*.

49 (original). The recombinant DNA of claim 47 wherein said DNA encodes at least one immunogenic epitope.

50 (original). A vector capable of expressing a recombinant DNA comprising:

a)a recombinant DNA inserted into said vector such that a recombinant protein is expressed when said vector is provided in an appropriate host wherein said DNA is selected from the group consisting of:

i) a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 3;

ii) a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 5;

iii) a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 7;

iv) a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 9; and

v) a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 11.

51- 65 (canceled).

Appl. No. 10/004,494
Declaration Of Yung-Fu Chang, Ph.D. Under 37 C.F.R. §1.132
Exhibit 3

INFECTION AND IMMUNITY, Apr. 2003, p. 2218–2225 Vol. 71, No. 4
0019-9567/03/$08.00+0 DOI: 10.1128/IAI.71.4.2218–2225.2003

Cloning and Characterization of an *Ehrlichia canis* Gene Encoding a Protein Localized to the Morula Membrane

Ching-Hao Teng, Raghavan U. M. Palaniappan, and Yung-Fu Chang*

Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, New York 14853

Received 1 November 2002/Returned for modification 26 November 2002/Accepted 17 December 2002

A gene encoding a 23.5-kDa ehrlichial morula membrane protein designated MmpA was cloned by screening an *Ehrlichia canis* expression library with convalescent dog sera, which resulted in three positive clones. Sequence analysis of the insert DNAs from all three clones indicated an open reading frame with a size of 666 bp that encodes MmpA. The structural analysis of MmpA indicated that it is a transmembrane protein with extreme hydrophobicity. Southern blot analysis of the *Hin*dIII-digested chromosomal DNA demonstrated the presence of a single copy of the *mmpA* gene in *E. canis* and *Ehrlichia chaffeensis* but not in the human granulocytic ehrlichiosis agent. The *mmpA* gene was amplified, cloned, and expressed as a fusion protein. Polyclonal antibodies to the recombinant protein (rMmpA) were raised in rabbits. Western blot analysis of *E. canis* and *E. chaffeensis* lysates with the anti-rMmpA serum resulted in the presence of an MmpA band only in *E. canis*, not in *E. chaffeenesis*. Sera from dogs which were either naturally or experimentally infected with *E. canis* recognized the recombinant protein. Double immunofluorescence confocal microscopy studies demonstrated that MmpA was localized mainly on the morula membrane of *E. canis*. Since the morula membrane is the interface between the ehrlichial growing environment and the host cytoplasm, MmpA may play a role in bacterium-host cell interactions.

Canine monocytic ehrlichiosis is a potentially fatal tick-borne disease caused by the rickettsia *Ehrlichia canis* (33). *E. canis* is an obligate intracytoplasmic rickettsia invading reticuloendothelial cells of the liver, spleen, and lymph nodes (33). The organism replicates primarily in monocytes and lymphocytes (33), and it is transmitted by the brown dog tick, *Rhipicephalus sanguineus*.

Donatien and Lestoquard first recognized canine monocytic ehrlichiosis in Algeria (17). Since then, it has been recognized worldwide as an important disease causing extensive morbidity and mortality among domestic dogs and other canids (24, 28, 33). In humans, the etiological agent of monocytic ehrlichiosis is *Ehrlichia chaffeensis* (31), whereas the causative organism of human granulocytic ehrlichiosis (HGE) was temporarily named the HGE agent (18, 23, 31) and in 2001 was named *Anaplasma phagocytophila* (18a). The phylogenetic analysis of 16S rRNA indicates that *E. canis* and *E. chaffeensis* have 98.2% homology (3). Western blot analysis of *E. canis* and *E. chaffeensis* lysates with antisera to *E. canis* and *E. chaffeensis* also revealed close antigenic similarity (14).

Similarly to all intracellular bacterial pathogens that form membrane-bound vesicles in the host cells, *E. canis* organisms form microcolonies inside cellular vacuoles (morulae) that harbor many individual ehrlichiae. Several survival strategies have been identified in various intracellular bacterial pathogens, such as escaping from vacuoles, avoidance of lysosomal fusion, and tolerance of the lysosomal environment (19). *Ehrlichia risticii*-containing morulae evade lysosomal fusion (44). The morula membrane provides a permissive environment not only for ehrlichial survival but also for replication. To date, several inclusion membrane proteins from *Chlamydia* spp. have been identified, but none in *E. canis* (5–7, 35, 37).

In order to identify ehrlichial antigens, an *E. canis* genomic library was constructed and screened with convalescent-phase dog sera. The screening resulted in the isolation of a gene encoding a protein that is localized on the morula membranes of *E. canis*-infected cells. The gene encoding this protein is named *mmpA* (for morula membrane protein A).

MATERIALS AND METHODS

Bacterial strains, plasmids, culture conditions, and an anti-*E. canis* monoclonal antibody (MAb). The *E. canis* Oklahoma strain and *E. chaffeensis* (ATCC CRL-10679) were cultured in the DH82 dog macrophage cell line (ATCC CCL-10389), and the HGE agent strain WI-1 was cultured in the HL-60 cell line (ATCC CCL-240) as previously described (15, 16, 23). Bacterial infection rates were determined by LeukoStat staining (Fisher Scientific, Pittsburgh, Pa.), and bacteria were counted under a microscope. *Escherichia coli* TB1 *hsdR* (27) and *E. coli* DH5α [F^- Φ80d *lac*Z ΔM15(*lacZYA-argF*) U169 *endA1 recA1 hsdR17* ($r_k^- m_k^+$) *deoR thi-1 phoA supE44λ-gyrA96 relA1*)] were used for plasmids pHG165 (38) and pRSETB (Invitrogen, Carlsbad, Calif.) manipulations. *E. coli* strain BL21(DE3)(pLysS) [F^- *ompT hsdS*$_B$ ($r_B^- m_B^-$) *dcm gal* λ(DE3) pLysS Cm^r] (Invitrogen) served as a host for the expression of the *mmpA* gene. *E. canis* was purified by Renografin gradient centrifugation as described elsewhere (42, 43).

An anti-*E. canis* MAb (anti-*E. canis*: 62.7-kDa protein mass [data not shown]) was a kind gift of Fort Dodge Laboratories, Fort Dodge, Iowa.

DNA manipulations. All standard DNA manipulations and analyses, except where mentioned, were performed according to the procedures described by Sambrook et al. (36).

Library construction and immunoscreening. *E. canis* DNA was extracted from purified organisms as previously described (11, 13). The purified DNA was subjected to *Sau*3A digestion, and the 3- to 8-kb fragments were isolated and ligated to plasmid pHG165 (38) and then transformed into *E. coli* TB1 (13). The recombinants were screened (colony blotting) with dog anti-*E. canis* antisera as previously described (13). Dog anti-*E. canis* antisera were prepared in beagles infected with live *E. canis* by intravenous injection as previously described (8, 41).

* Corresponding author. Mailing address: Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853. Phone: (607) 253-3675. Fax: (607) 253-3943. E-mail: yc42@cornell.edu.

These beagles were checked by detection of morulae in the monocytes. The antiserum was preabsorbed with *E. coli* TB1 lysates before use.

Southern blot analysis. *E. canis*, *E. chaffeensis*, and the HGE agent genomic DNAs were prepared as previously described (11, 13), digested with *Hin*dIII, electrophoresed through a 0.7% agarose gel, transferred to nitrocellulose membranes, and probed with a 427-bp DNA fragment containing the *mmpA* gene amplified by PCR with a primer pair (1RACE1, 5′-GCTGCATTCTTGTTTGC TGC-3′, and 4F, 5′-ACGTGAGTTTGTTTATCTGGAC-3′) (see Fig. 2). The DNA fragment was labeled with a nonradioactive labeling kit (ECL direct nucleic acid labeling and detection systems; Amersham, Little Chalfont, Buckinghamshire, England) (12). Southern blot hybridization and detection were performed as described by the manufacturer.

PCR procedures and subcloning of *mmpA* gene. We designed a primer pair to amplify the whole *mmpA* gene with the exception of the first 63 bp. The primer pair consisted of a sense primer, EC2F3 (5′-CAGAATTCGCAGTGTTAGGT TTAGCT-3′) and an antisense primer, EC2R2 (5′-GCAAGCTTAGGTGAAT ACAGGCTAAA −3′) (see Fig. 2). PCR was carried out in a Perkin-Elmer Gene Amp PCR system 9600 thermal cycler. The amplification reaction was performed in a final volume of 50 μl containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.4 mM (each) deoxynucleoside triphosphate (Pharmacia, Piscataway, N.J.), primers (0.2 μM), 1.25 U of *Taq* polymerase (Gibco BRL, Grand Island, N.Y.), 1 μl of template, and 33 μl of distilled water. The template (pCH2) was denatured at 94°C for 30 s, and 30 amplification cycles were performed as follows: 30 s of denaturation at 94°C, 45 s of annealing at 56°C, and 30 s of primer extension at 72°C, followed at 72°C for 15 min and held at 4°C. The PCR product containing the *mmpA* gene was cut with *Eco*RI and *Hin*dIII and ligated to pRSETB cut with the same restriction enzymes (Invitrogen) and was designated pTEC2. pTEC2 was transferred into *E. coli* strain BL21(DE3)(pLysS).

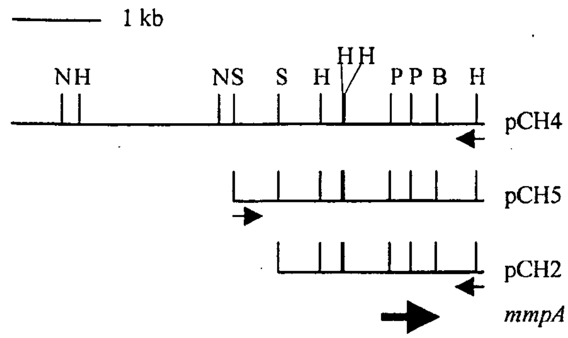

FIG. 1. Ehrlichial DNA inserts in pCH4, pCH5, and pCH2 with their restriction map. The location and direction of the *mmpA* gene is indicated by the large arrow. The direction of the *lac* promoter from plasmid pHG165 is indicated by small arrows. B, *Bam*HI; H, *Hin*dIII; N, *Nde*I; P, *Pst*I; and S, *Sau*3A.

Purification of MmpA protein and antiserum production in rabbits. *E. coli* BL21(DE3)(pLysS) harboring pTEC2 was grown in Luria-Bertani medium to an optical density at 600 nm of ~0.5. IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 1 mM, and the culture was grown for 3 h. The cells were harvested by centrifugation and resuspended in phosphate-buff-

```
          CTTAATAGTATAGCTGTTGACAAGCCGCAATCTGCGGTTC
                                                ^^^^^^
-60 TTGACAAAATAATACTAATCAGTTAAAATTTTGAAGTGTTTCACCATAATGGTATTATTT
     -35                    -10
  1 ATGAAAGCTCATAGCACAAGTATACGGAACTTTCAGCCTTTAGAAAGAGCTGCTATAATC
     M  K  A  H  S  T  S  I  R  N  F  Q  P  L  E  R  A  A  I  I
 61 ATTGCAGTGTTAGGTTTAGCTGCATTCTTGTTTGCTGCTGCTGCCTGCAGTGATCGTTTC
     I  A  V  L  G  L  A  A  F  L  F  A  A  A  A  C  S  D  R  F
121 CAAAGATTGCAATTAACAAATCCATTTGTAATAGCAGGAATGGTTGGCCTTGCAGTTCTT
     Q  R  L  Q  L  T  N  P  F  V  I  A  G  M  V  G  L  A  V  L
181 TTAGTTGCTTCCTTAACAGCAGCATTAAGTATATGCTTAACTAAAAGTAAGCAAGTCACA
     L  V  A  S  L  T  A  A  L  S  I  C  L  T  K  S  K  Q  V  T
241 CAACATGCTATTAGACATCGCTTTGGATACGAGTCAAGCACTTCTTCTTCTGTACTGCTT
     Q  H  A  I  R  H  R  F  G  Y  E  S  S  T  S  S  S  V  L  L
301 GCAATATCAATAATTTCTTTATTACTTGCTGCAGCATTTTGTGGAAAGATAATGGGTAAT
     A  I  S  I  I  S  L  L  L  A  A  A  F  C  G  K  I  M  G  N
361 GACAACCCAGATCTATTCTTTAGCAAGATGCAAGAACTCTCCAATCCACTTGTTGTTGCA
     D  N  P  D  L  F  F  S  K  M  Q  E  L  S  N  P  L  V  V  A
421 GCTATTGTAGCCGTTTCTGTTTTCCTACTCTCATTCGTAATGTATGCTGCAAAGAACATT
     A  I  V  A  V  S  V  F  L  L  S  F  V  M  Y  A  A  K  N  I
481 ATAAGTCCAGATAAACAAACTCACGTTATTATATTATCTAATCAACAAACTATAGAAGAA
     I  S  P  D  K  Q  T  H  V  I  I  L  S  N  Q  Q  T  I  E  E
541 GCAAAAGTAGATCAAGGAATGAATATTTTGTCAGCAGTACTCCCAGCAGCTGGCATTGAC
     A  K  V  D  Q  G  M  N  I  L  S  A  V  L  P  A  A  G  I  D
601 ATCATGACTATAGCTTCTTGTGACATTTTAGCAGTGAGCAGCCGGGGATCCTCTCAGCAT
     I  M  T  I  A  S  C  D  I  L  A  V  S  S  R  G  S  S  Q  H
661 CAATAGATTTATGTTTTAGCCTGTATTCACCTTTTTATTAGGTGTTGTATCGTTTCTTTA
     Q  *                                                   ----
721 TATAAGTGTGTTATATTATATAAAACAT
    ---->         <--------
```

FIG. 2. Nucleotide sequences of the *mmpA* gene and its coded protein. Nucleotide numbers are indicated on the left. The start and stop codons are indicated in boldface type. A promoter-like region proximal to *mmpA* is underlined. The potential ribosome-binding site preceding *mmpA* is indicated by carets. A potential transcription terminator of *mmpA* is indicated by dashed arrows. The underlined nucleotides indicate the primer annealing sites for generation of an *mmpA* gene fragment for insertion into pRESTB. The italicized nucleotides indicate the primer annealing sites for the generation of the DNA probes used for Fig. 4. The stop codon (amino acid) is indicated by an asterisk.

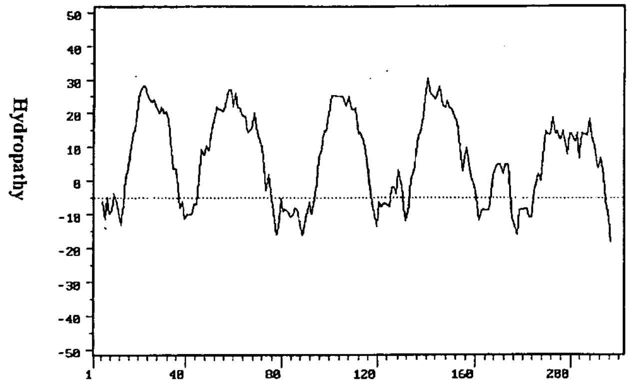

FIG. 3. Hydropathy plot of the predicted amino acids of MmpA. The vertical axis represents the scale of the hydrophobic (positive) and hydrophilic (negative) values established for each window of nine amino acids as previously described (30).

ered saline (PBS) containing 0.5% Triton X-100 and 10 mM EDTA. The cells were ruptured by a French press at 8,000 lb/in^2. The total lysate was then spun at 3,000 × g for 5 min to separate cell debris (10) and then centrifuged at 12,000 × g for 5 min at 4°C. The pellets were washed with 9 volumes of PBS containing 0.5% Triton X-100 and 10 mM EDTA, incubated at room temperature for 5 min, and then centrifuged at 12,000 × g for 5 min at 4°C. The washing step was repeated once. The protein samples (~500 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were visualized after being stained with Coomassie blue. The recombinant MmpA (rMmpA) band was excised, mixed with an equal volume of PBS, and ground to homogeneity for injection into rabbits. Polyclonal antiserum to rMmpA was raised in New Zealand White rabbits by three subcutaneous injections at 3-week intervals with ~100 μg of rMmpA in Freund's incomplete adjuvant as previously described (13).

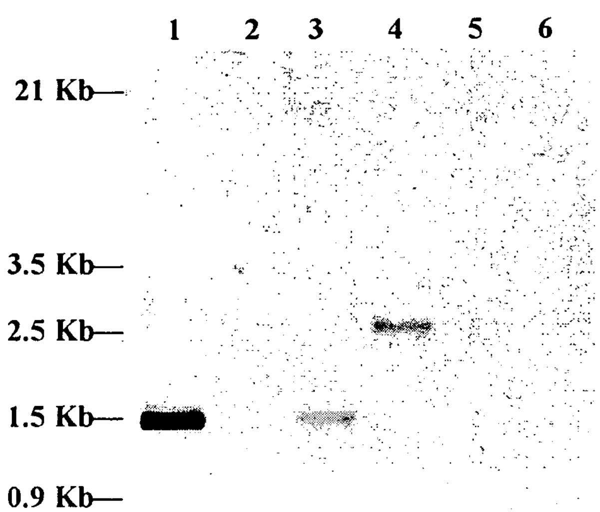

FIG. 4. Southern blot hybridization with the PCR products (427 bp) derived from *mmpA* against total genomic DNA digests (~20 μg of chromosomal DNA was digested with *Hin*dIII) from DH-82 cells (lane 2), *E. canis*-infected cells (lane 3), *E. chaffeensis*-infected DH-82 cells (lane 4), HL-60 cells (lane 5), and HGE agent-infected HL-60 cells (lane 6). pCH4 digested with *Hin*dIII, served as a positive control (lane 1).

SDS-PAGE and Western and dot blotting. The procedures for the SDS-PAGE and Western blot analyses were as previously described (9, 11). For dot blotting, *E. canis*- or *E. chaffeensis*-infected DH82 cells (4 to 5 days old with an ~50 to 60% infection rate) were gently scraped from the bottom of a 25-cm^2 flask (6 ml) with a rubber policeman. The cells were pelleted (6,000 × g; 10 min), washed twice with PBS (pH 7.2), resuspended with 1 ml of PBS, and subjected to sonication at setting 4 for 2 min on ice (model W-185 Sonifier; Branson Ultrasonics Corp., Danbury, Conn.). Unbroken cells were removed by centrifugation at 1,000 × g for 10 min at 4°C, and the lysate was subjected to dot blot analysis. For identification of rMmpA expression, MAbs against the Xpress epitope (Invitrogen) were used as primary antibodies. The secondary antibody, alkaline phosphatase-conjugated affinity-purified anti-mouse immunoglobulin G (IgG) (KPL, Gaithersburg, Md.) was used at a dilution of 1:5,000. For testing the expression of MmpA in *E. canis* and of possible MmpA homologues in *E. chaffeensis* and HGE, the rabbit anti-rMmpA antiserum served as the primary antibody (1:500). Goat alkaline phosphatase-conjugated anti-rabbit IgG (KPL) was used as a secondary antibody (1:5,000). To determine whether the naturally and experimentally infected dog sera contained anti-MmpA antibodies, rMmpA protein (~1 μg/lane) was used as an antigen and subjected to SDS-PAGE and Western blot analysis. Test sera (1:500) from experimentally or naturally infected animals were used as the first antibody, followed by goat anti-dog IgG conjugated to alkaline phosphatase (1:5,000) (KPL) as the second antibody.

Location of the MmpA protein in *E. canis*. To localize the MmpA protein, *E. canis* was cultured on DH82 cells and subjected to double immunofluorescence labeling and confocal microscopy (MRC 600; Bio-Rad, Hercules, Calif.) examination. Briefly, *E. canis*-infected DH82 cells (~60 to 70% infected) 4 to 5 days after infection were gently scraped from the bottom of a 25-cm^2 flask (6 ml) with a rubber policeman. The cells were harvested by centrifugation at 6,000 × g for 10 min and resuspended with 2 ml of fresh culture medium. The cell suspensions (5 μl) were loaded into individual wells of 12-well Teflon-coated slides (Erie Scientific, Portsmouth, N.H.), left to air dry, and fixed in acetone for 15 min. The slides were maintained at −20°C until they were used.

For the first immunofluorescence labeling, the frozen slides were air dried again and then fixed in methanol for 3 min. The fixed cells were incubated with 20 μl of rabbit antiserum to MmpA in each well at a 1:100 dilution (PBS with 0.05% SDS and 5% fetal calf serum) for 30 min at 37°C. After incubation, the slides were washed by dipping them twice into PBS followed by immersion in fresh PBS for 5 min and then were washed twice with distilled water and air dried. Fluorescein isothiocyanate-conjugated anti-rabbit IgG (Sigma, St. Louis, Mo.), a second antibody, was prepared at a 1:30 dilution with the same buffer used for the primary antibody. The labeling process for the second antibody was similar to that for the primary antibody.

For the second immunofluorescence labeling, after the steps above, the cells were sequentially incubated with the anti-*E. canis* MAb and then with lissamine-rhodamine-conjugated anti-mouse IgG (Jackson) in PBS with 0.05% Tween 20 and 5% fetal calf serum at a 1:40 dilution. All other conditions were identical to the procedures described above for the first immunofluorescence labeling. After all the procedures, the slides were air dried and mounted with mounting fluid (2.5 μl/well; Becton Dickinson, Sparks, Md.). The tests were repeated five times. Each time, 100 morulae were counted.

5′-RACE. To determine the transcription site, 5′ rapid amplification of cDNA ends (5′-RACE) was followed as previously described (21) with the following modifications. Briefly, RNA was extracted by using RNAzol (Tel-test, Friendswood, Tex.), and the first-strand cDNA (antisense) was synthesized by using an *mmpA*-specific primer (2RACE1, 5′-CTTTCCACAAAATGCTGCAG-3′). The RNA template was then degraded with RNase H (BRL, Life Technologies, Rockville, Md.), and single-stranded cDNA was purified with the PRC purification kit (Gibco BRL, Life Technologies, Rockville, Md.). An oligo(dC) anchor sequence was added to the 3′ end of the cDNA using terminal deoxynucleotidyltransferase (TdT) and dCTP. PCR amplification was accomplished with two primers: a deoxynosine-containing anchor primer (Qc, 5′-CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCTTGGGIIGGGIIGGGIIG-3′) that annealed to the poly(C) tail of the cDNA and a nested *mmpA*-specific primer (2RACE2, 5′-TTGCAAGCAGTACAGAAGAAG-3′). Following that, a second-round nested PCR was performed with primer Qo (5′-CCAGTGAGCAGAGTGACG-3′), which was complementary to the 5′ end of Qc, and the other *mmpA*-specific primer (2RACE3, 5′-GTGCTTGACTCGTATCCAAAG-3′). The resulting PCR product was cloned in a TA cloning vector (Invitrogen) and subjected to DNA sequencing. We repeated 5′-RACE three times, and one clone from each repetition was subjected to DNA sequencing.

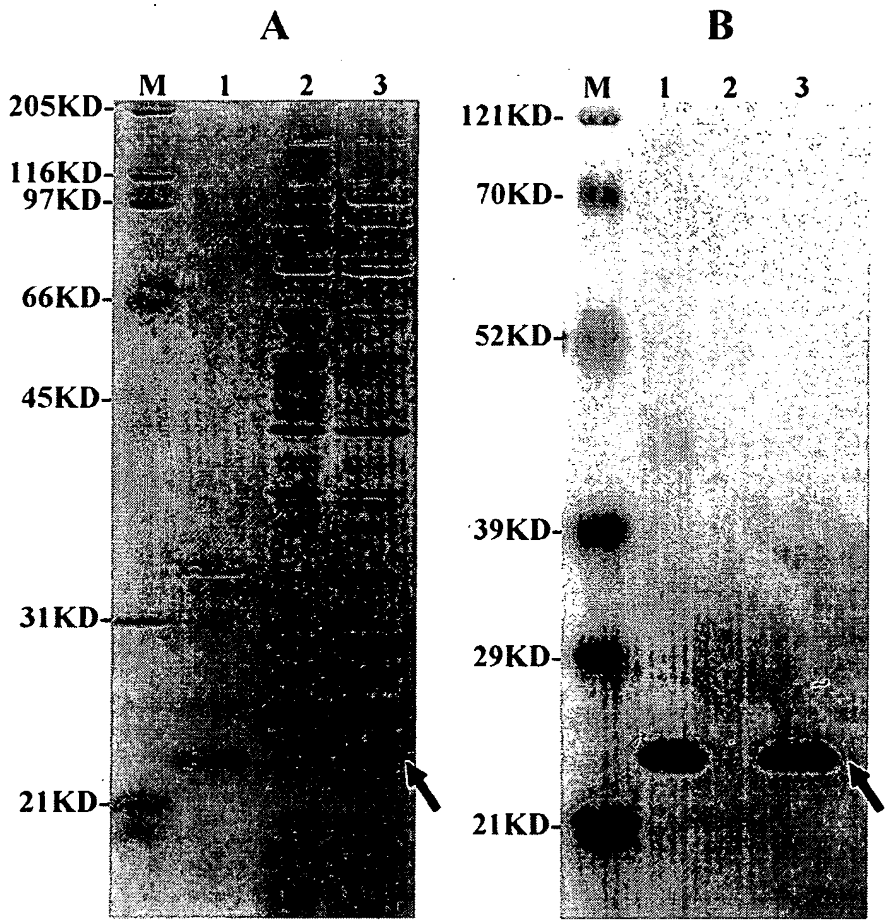

FIG. 5. Identification of rMmpA by SDS-PAGE (A) and Western immunoblot assay with MAb against the Xpress epitope (B). Lanes M, mass marker protein; lanes 1, partially purified rMmpA (inclusion bodies); lanes 2, *E. coli* lysate (negative control); lanes 3, IPTG-induced pTEC2-transformed *E. coli* lysate. The expressed protein band is indicated by an arrow.

DNA sequencing and analysis. Automated DNA sequencing was performed on an Applied Biosystems model 373A DNA system by using the *lac* universal primer and primers complementary to regions already sequenced in the plasmid. The thermal cycling of the sequencing reactions utilized the *Taq* DyeDeoxy Terminator cycle-sequencing kit. Both strands of the cloned DNA were completely sequenced. The nucleotide sequence was analyzed using the BLAST programs of the National Center for Biotechnology Information (NCBI) (1, 2).

Nucleotide sequence accession number. The GenBank accession number of the nucleotide sequence of pCH4 determined in this study is AF219120.

RESULTS

Immunoscreening of an *E. canis* genomic library. A plasmid library of *E. canis* genomic DNA was screened using pooled convalescent dog sera, and three clones that reacted strongly to the antisera were identified. These clones contained the plasmids pCH2, pCH4, and pCH5 with DNA insertions of 2.4, 2.8, and 5.3 kbp, respectively (Fig. 1).

Sequence analysis of *mmpA* genomic clones. The *E. canis* DNAs in pCH2, -4, and -5 were sequenced, and the results revealed the presence of an overlapping open reading frame (ORF) in all three clones. This ORF, with a size of 666 bp, was designated *mmpA* (Fig. 2).

The DNA sequence of *mmpA* was searched for *E. coli* promoter consensus sequences using the homology score method (32). Putative −10 (TAAAAT) and −35 (TTGACA) promoter regions were identified in an upstream region of the initiation codon (Fig. 2). A ribosome-binding site (Shine-Dalgarno sequence) was also evident upstream of the initiation codon, and an inverted repeat sequence that is probably capable of forming a stem-loop ($\Delta G = -7.4$ kCal/mol) and thus terminating transcription was also found downstream of the stop codon of *mmpA* (Fig. 2).

The deduced amino acid sequence was also used to search for protein homologues in the NCBI database, but there was no significant homology to any other proteins in the database. The hydrophobicities of the deduced amino acids of MmpA and its potential transmembrane regions were analyzed using previously described methods (30), and the analysis predicted MmpA to be extremely hydrophobic, with five main potential transmembrane regions (Fig. 3). Based on the method developed by Hopp and Woods, three potential antigenic determinants were identified (25). Three potential protein kinase C phosphorylation sites located in positions 7 (Ser), 37 (Ser), and 213 (Ser) and one possible casein kinase II phosphorylation site in position 177 (Thr) were found by using the program PROSITE (PCgene), as previously described (20).

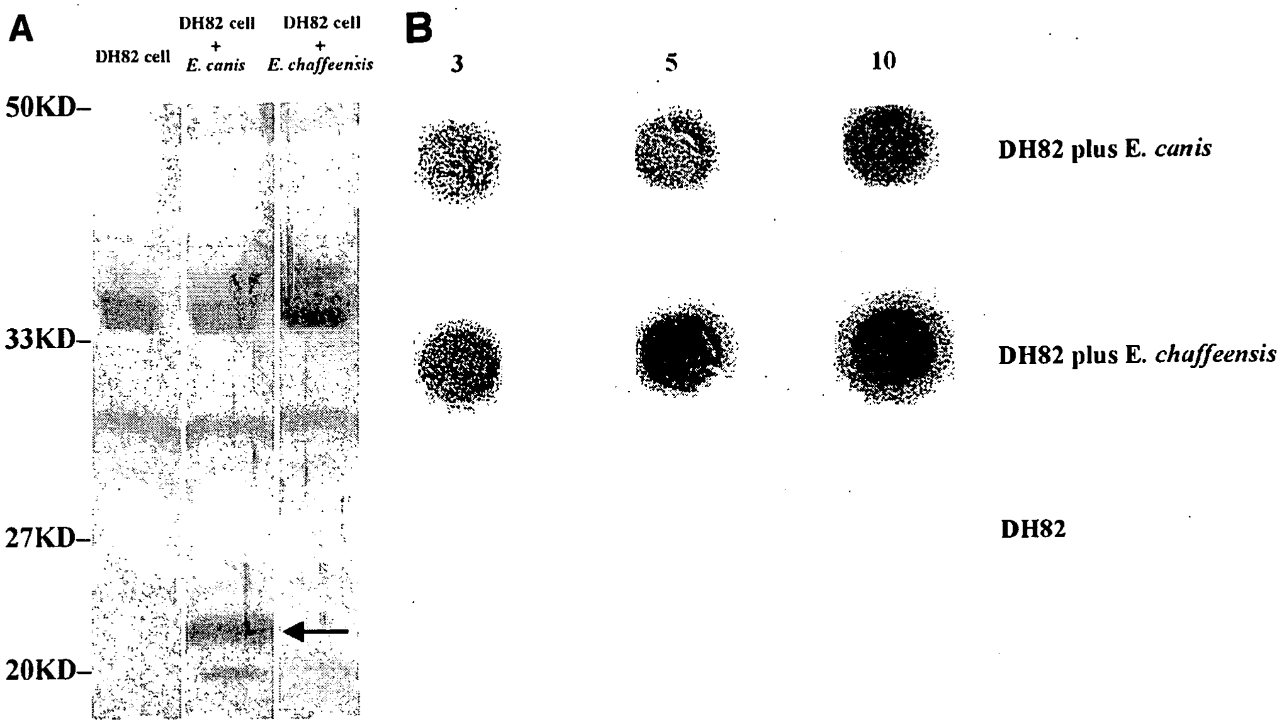

FIG. 6. Western (A) and dot (B) immunoblot assays of DH82 cells, *E. canis*-infected DH82 cells, and *E. chaffeensis*-infected DH82 cells with rabbit anti-rMmpA serum. The molecular masses of markers (Bio-Rad) are indicated on the left. For dot blot analysis (Bio-Dot; Bio-Rad), 3, 5, and 10 μl of lysate (see Materials and Methods) were adjusted to 10 μl using PBS and subjected to analysis following the manufacturer's instructions.

Southern blot analysis. To address the frequency and distribution of the *mmpA* gene in *E. canis*, *E. chaffeensis*, and the HGE agent, Southern blot analyses of *Hin*dIII-digested genomic DNAs of *E. canis*-infected DH82 cells, *E. chaffeensis*-infected DH82 cells, and HGE agent-infected HL60 cells were performed. The digested genomic DNAs from DH82 and HL60 cells were used as negative controls, and pCH4 was used as a positive control. A single band with a size of 1.5 kb was detected from *E. canis*-infected DH82 cells (Fig. 4, lane 3) and pCH4 (Fig. 4, lane 1), whereas a 2.5-kb band was detected from *E. chaffeensis*-infected DH82 cells (Fig. 4, lane 4). The HGE agent-infected HL60 cells (Fig. 4, lane 6) and controls, such as DH82 and HL60 cells (Fig. 4, lanes 2 and 5), did not show any bands. The results suggest that the *mmpA* gene is present as a single copy in *E. canis* and *E. chaffeensis* but is not present in the HGE agent.

5′-RACE for determination of the transcriptional start site. To determine the transcriptional start site of *mmpA*, 5′-RACE was performed. The cDNA product was cloned in a TA cloning vector and subjected to DNA sequence analysis. We repeated RACE three times, and one clone from each repetition was subjected to DNA sequencing. Based on the sequencing results, the transcriptional start site was located at either a G or a T 7 or 8 nucleotides downstream from the promoter −10 region, respectively.

Expression of MmpA and Western blotting analysis. A 0.6-kb *mmpA* gene fragment was amplified and cloned into pRSETB as described in Materials and Methods. This construct (pTEC2) was transferred into *E. coli* and expressed as a recombinant protein (rMmpA). rMmpA contains a six-His Tag and an Xpress epitope on its N terminus. A partial purification of the recombinant protein from the *E. coli* lysate was accomplished by washing it with Triton X-100 buffer, since the molecule formed insoluble inclusion bodies. The molecular mass of the partially purified recombinant protein was found by SDS-PAGE to be ~26 kDa (Fig. 5A). Western blot analysis with anti-Xpress antibodies also confirmed the in-frame fusion of the His tag and rMmpA (Fig. 5, B).

To determine whether *E. chaffeensis* expressed MmpA, lysates from *E. canis*-infected DH82 cells, *E. chaffeensis*-infected DH82 cells, and uninfected DH82 cells (negative control) were probed with anti-rMmpA antibodies in a Western blot analysis. The results showed only a protein band of ~23.5 kDa closely correlated with the deduced molecular mass of MmpA in *E. canis*-infected cells. No protein band was detected from either *E. chaffeensis*-infected DH82 cells or uninfected DH82 cells by anti-MmpA antibodies (Fig. 6A). However, dot blot analysis indicated the presence of an MmpA homologue in *E. chaffeensis*-infected DH82 cells (Fig. 6B).

To evaluate whether naturally and experimentally infected dog sera contained anti-MmpA antibodies, four naturally and one experimentally infected dog serum plus one uninfected and two specific-pathogen-free (SPF) dog sera were used for Western blot analysis. The results showed that partially purified rMmpA was recognized by all the naturally and experimentally *E. canis*-infected dog antisera, but not by the normal serum or the SPF dog sera (Fig. 7). These data suggest in vivo

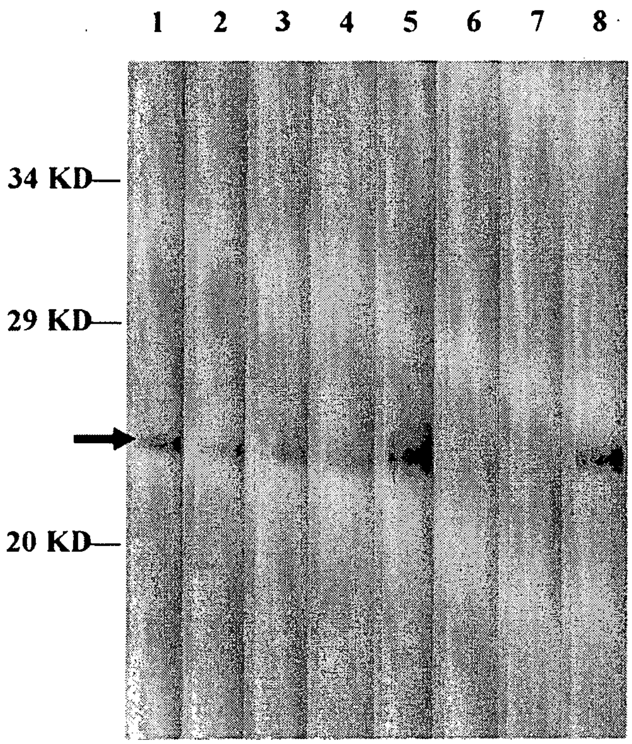

FIG. 7. Western blot of sera obtained from dogs experimentally or naturally infected with *E. canis*. The lanes show reactivity with rMmpA. Serum from a dog experimentally infected with *E. canis* (lane 1), sera from four different naturally *E. canis*-infected dogs (lanes 2 to 5), sera from two SPF dogs (lanes 6 and 7), and pooled convalescent-phase sera (lane 8) were used to screen the library.

expression of MmpA and its involvement in canine ehrlichiosis.

Subcellular localization of MmpA. The intracellular location of MmpA was determined by using anti-rMmpA antibodies and anti-*E. canis* MAbs for double immunofluorescence labeling of *E. canis*-infected DH82 cells under confocal microscopy. The results revealed that the MmpA antigens were detectable mostly on the morula membrane, displaying a nonuniform fluorescent-staining pattern in ~80% of the morulae examined (Fig. 8). Repeated attempts with increasing or decreasing concentrations of primary or secondary antibodies or affinity-purified primary antibodies could not eliminate the background staining, but the prominent staining of the morula membrane suggests that the MmpA antigen is mainly located on the morula membrane (Fig. 9). In contrast, the MAb stained only the *E. canis* organisms within the morulae. Normal rabbit serum (preimmune rabbit serum) did not stain either infected or uninfected DH82 cells (data not shown).

DISCUSSION

Ehrlichia spp. reside mainly in monocytes, macrophages, and neutrophils, which are considered primary effector cells of antimicrobial defense. Within the host cell, the bacteria reside within inclusion bodies (morulae), which provide a hospitable environment for their survival. The intracellular events leading to the establishment and maintenance of ehrlichia morulae in the host cells, especially for *E. canis*, is poorly understood. In this study, we have identified and partially characterized an ehrlichial morula membrane protein, MmpA, which was obtained by screening an *E. canis* genomic library with convalescent-phase dog sera. Only three positive clones were identified, and all of them contained an ORF that encodes MmpA. Neither the *mmpA* gene nor the MmpA protein shows any significant homology with other genes or proteins in the NCBI databases. The structural analysis of MmpA predicts that it is extremely hydrophobic, with five transmembrane segments (Fig. 3) and three potential antigenic determinants. We found that in vitro-grown (DH82 cells) *E. canis* expressed MmpA. In addition, the sera obtained from dogs naturally and experimentally infected with *E. canis* recognized MmpA. Taken together, these results confirm not only the antigenicity but also the in vivo and in vitro expression of MmpA.

Repeated attempts with 5′-RACE indicated that the *mmpA* transcription start site was either a G or T located downstream of the −10 promoter. Since the G is located at the corresponding G of the template, and also adjacent to the appended poly(G) tail, it is difficult to say whether the G is derived from poly(C) addition during 5′-RACE or from the cDNA. However, this G or T is in good agreement with the consensus spacing of 6 to 8 bp between the promoter and the transcription start site.

We found the *mmpA* gene in *E. canis* and *E. chaffeensis* but not in the HGE agent. This is not surprising, since there is a higher homology between *E. canis* and *E. chaffeensis* than be-

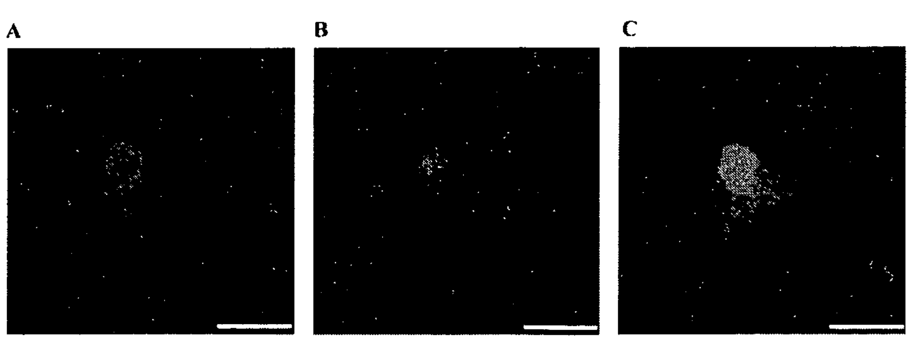

FIG. 8. (A and B) Confocal micrographs of a single plane (0.5 μm thick) showing the localization of MmpA antigen on the morula membrane by probing with anti-rMmpA serum (A) and a MAb recognized by *E. canis* antigen in the organisms (B). (C) Merged images from panels A and B. Antibody labels: MmpA (green), anti-*E. canis* antigen (red). Bars = 18.5 μm.

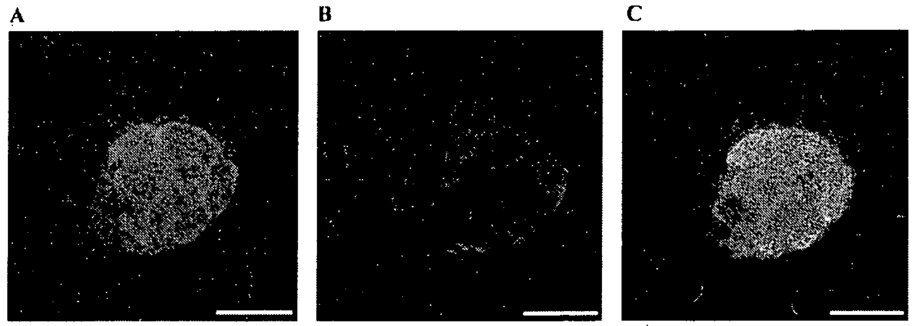

FIG. 9. (A and B) Confocal micrographs of a single plane (0.5 μm thick) showing the localization of MmpA antigen on the morula membrane by probing with anti-rMmpA serum (A) and a MAb recognized by *E. canis* antigen in the organisms (B). (C) Merged images from panels A and B. Antibody labels: MmpA (green), anti-*E. canis* antigen (red). Bars = 18.5 μm.

tween *E. canis* and the HGE agent, according to either 16S rRNA or *groEL* heat shock gene analysis (39, 40). Since this gene is not present in the HGE agent, it may serve as a genetic marker to differentiate the HGE agent from the above-mentioned monocytic ehrlichiae. However, this conclusion must be viewed cautiously, since we have examined only one strain of each organism. Western blot analysis of lysates from *E. canis* and *E. chaffeensis* with anti-MmpA sera demonstrated the presence of MmpA in *E. canis* at the predicted molecular mass of 23.5 kDa but not in *E. chaffeensis* (Fig. 6A). However, the dot blot analysis revealed the presence of an MmpA-like protein in *E. chaffeensis* culture (Fig. 6B), and it indicates that SDS may denature the MmpA homologue during Western blot analysis. Further cloning and sequencing of the *E. chaffeensis* homologous gene is needed to answer this question.

Confocal fluorescence microscopy of *E. canis*-infected cells revealed that MmpA was localized primarily in the morula membrane. Several attempts to determine the subcellular location of the MmpA protein by immunogold labeling were unsuccessful (unpublished data). Since the MmpA appears to be localized on the morula membrane, it is likely to require transport across the ehrlichial membranes. The secretory pathways for some of the intracellular pathogens have been elucidated, but none has been reported in *Ehrlichia* spp. (4, 10, 22, 26). Based on the predicted amino acid sequence, MmpA may not use a classic type II secretion system, because it lacks the typical type II signal sequence at its amino terminus. Several other secretion systems, such as type III, which do not require N-terminal signal sequences may be involved in MmpA secretion. The secretory pathways used by the ehrlichiae await elucidation.

To our knowledge, MmpA is the first *E. canis* protein to be found on the morula membrane. A 44-kDa protein in the HGE agent, a member of the genus *Ehrlichia*, is the only other protein that has been reported to partially localize on the morula membrane (29). Several other proteins that are also localized in inclusion membranes have been identified in other intracellular bacteria. For example, in *Chlamydia psittaci* and *Chlamydia trachomatis*, three products of three genes, *incA*, *-B*, and *-C*, have been shown to localize to the inclusion membrane. The deduced amino acid sequences of these genes show less similarity to each other, but all of them have a common bilobed hydrophobic motif (6, 37). Based on the hydrophobic motif of Inc proteins, Bannantine et al. recently predicted that there are probably 46 inclusion membrane proteins in *C. trachomatis* (5). In contrast to chlamydia proteins, the MmpA protein of *E. canis* contains five major hydrophobic domains, and this motif may serve as a model to find other morula membrane proteins. Furthermore, we identified a putative operon with two genes (probably encoding endopeptidases) upstream of *mmpA*, while downstream of this gene is a probable lipoprotein signal peptidase gene (this study). Likewise, in chlamydia, the regions upstream of the *incA*, *-B*, and *-C* genes have two ORFs encoding 40- and 44-kDa proteins that are similar to amino acid transporters and Na^+-dependent transporters, respectively (6). The function of MmpA, together with those of most of the identified chlamydial inclusion membrane proteins, remains to be characterized. Phosphorylation of one of the chlamydial inclusion membrane proteins, IncA, by the host cells indicates communication between the bacteria and the host cells (34). MmpA also possesses three potential phosphorylation sites, indicating the possibility of a similar communication strategy between *E. canis* and host cells.

In conclusion, we have identified and expressed a novel ehrlichial gene (*mmpA*) coding for a protein (MmpA) that is localized on the morula membrane of *E. canis*. This gene is present in *E. canis* and *E. chaffeensis* but not in the agent of HGE. However, the encoded protein is detected only in *E. canis*. Since it is an immunodominant protein, it may also be a candidate for recombinant or DNA vaccine development and/or a serologic reagent. Further characterization of MmpA function may offer significant insights into the molecular mechanisms of ehrlichial pathogenesis.

ACKNOWLEDGMENTS

This work was supported by grants from Fort Dodge Animal Health, a Division of American Home Products Corporation, and the New York State Science and Technology Foundation.

REFERENCES

1. **Altschul, S. F., and E. V. Koonin.** 1998. Iterated profile searches with PSI-BLAST—a tool for discovery in protein databases. Trends Biochem. Sci. **23:**444–447.
2. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
3. **Anderson, B. E., J. E. Dawson, D. C. Jones, and K. H. Wilson.** 1991. *Ehrlichia chaffeensis*, a new species associated with human ehrlichiosis. J. Clin. Microbiol. **29:**2838–2842.

4. **Balakrishnan, L., C. Hughes, and V. Koronakis.** 2001. Substrate-triggered recruitment of the TolC channel-tunnel during type I export of hemolysin by *Escherichia coli*. J. Mol. Biol. **313**:501–510.
5. **Bannantine, J. P., R. S. Griffiths, W. Viratyosin, W. J. Brown, and D. D. Rockey.** 2000. A secondary structure motif predictive of protein localization to the chlamydial inclusion membrane. Cell Microbiol. 2:35–47.
6. **Bannantine, J. P., D. D. Rockey, and T. Hackstadt.** 1998. Tandem genes of *Chlamydia psittaci* that encode proteins localized to the inclusion membrane. Mol. Microbiol. **28**:1017–1026.
7. **Bannantine, J. P., W. E. Stamm, R. J. Suchland, and D. D. Rockey.** 1998. *Chlamydia trachomatis* IncA is localized to the inclusion membrane and is recognized by antisera from infected humans and primates. Infect. Immun. **66**:6017–6021.
8. **Buhles, W. C., D. L. Huxsoll, and M. Ristic.** 1974. Tropical canine pancytopenia: Clinical, hematologic, and serologic response of dogs to *Ehrlichia canis* infection, tetracycline therapy, and challenge inoculation. J. Infect. Dis. **130**:357–367.
9. **Chang, Y. F., M. J. Appel, R. H. Jacobson, S. J. Shin, P. Harpending, R. Straubinger, L. A. Patrican, H. Mohammed, and B. A. Summers.** 1995. Recombinant OspA protects dogs against infection and disease caused by *Borrelia burgdorferi*. Infect. Immun. **63**:3543–3549.
10. **Chang, Y.-F., T.-L. Lauderdale, W. Y. Lee, S. J. Shin, R. H. Jacobson, M. J. Appel, and D. H. Lein.** 1992. Expression and secretion of outer surface protein (Osp-A) of *Borrelia burgdorferi* from *Escherichia coli*. FEMS Microbiol. Lett. **109**:297–302.
11. **Chang, Y. F., J. Shi, D. P. Ma, S. J. Shin, and D. H. Lein.** 1993. Molecular analysis of the *Actinobacillus pleuropneumoniae* RTX toxin-III gene cluster. DNA Cell Biol. **12**:351–362.
12. **Chang, Y.-F., R. K. Straubinger, R. H. Jacobson, J. B. Kim, T. J. Kim, D. Kim, S. J. Shin, and M. J. G. Appel.** 1996. Dissemination of *Borrelia burgdorferi* after experimental infection in dogs. J. Spiroch. Tick-borne Dis. 3:80–86.
13. **Chang, Y. F., R. Young, D. Post, and D. K. Struck.** 1987. Identification and characterization of the *Pasteurella haemolytica* leukotoxin. Infect. Immun. **55**:2348–2354.
14. **Chen, S. M., J. S. Dumler, H. M. Feng, and D. H. Walker.** 1994. Identification of the antigenic constituents of *Ehrlichia chaffeensis*. Am. J. Trop. Med. Hyg. **50**:52–58.
15. **Dawson, J. E., B. E. Anderson, D. B. Fishbein, J. L. Sanchez, C. S. Goldsmith, K. H. Wilson, and C. W. Duntley.** 1991. Isolation and characterization of an *Ehrlichia* sp. from a patient diagnosed with human ehrlichiosis. J. Clin. Microbiol. **29**:2741–2745.
16. **Dawson, J. E., F. J. Candal, V. G. George, and E. W. Ades.** 1993. Human endothelial cells as an alternative to DH82 cells for isolation of *Ehrlichia chaffeensis*. *E. canis*, and *Rickettsia rickettsii*. Pathobiology **61**:293–296.
17. **Donatien, A., and F. Lestoquard.** 1935. Existence en Algerie d'une Rickettsia du chien. Bull. Soc. Pathol. Exot. **28**:418–419.
18. **Dumler, J. S., and J. S. Bakken.** 1996. Human granulocytic ehrlichiosis in Wisconsin and Minnesota: a frequent infection with the potential for persistence. J. Infect. Dis. **173**:1027–1030.
18a. **Dumler, J. S., A. F. Barbet, C. P. Bekker, G. A. Dasch, G. H. Palmer, S. C. Ray, Y. Rikihisa, and F. R. Rurangirwa.** 2001. Reorganization of genera in the families *Rickettsiaceae* and *Anaplasmataceae* in the order *Rickettsiales*: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. Int. J. Syst. Evol. Microbiol. **51**:2145–2165.
19. **Ernst, R. K., T. Guina, and S. I. Miller.** 1999. How intracellular bacteria survive: surface modifications that promote resistance to host immune responses. J. Infect. Dis. **179**:S326-S330.
20. **Falquet, L., M. Pagni, P. Bucher, N. Hulo, C. J. Sigrist, K. Hofmann, and A. Bairoch.** 2002. The PROSITE database. its status in 2002. Nucleic Acids Res. **30**:235–238.
21. **Frohman, M.** 1993. Rapid amplification of complementary DNA ends for generation of full-length complementary DNAs: thermal RACE. Methods Enzymol. **218**:340–356.
22. **Galan, J. E., and A. Collmer.** 1999. Type III secretion machines: bacterial devices for protein delivery into host cells. Science **284**:1322–1328.
23. **Goodman, J. L., C. Nelson, B. Vitale, J. E. Madigan, J. S. Dumler, T. J. Kurtti, and U. G. Munderloh.** 1996. Direct cultivation of the causative agent of human granulocytic ehrlichiosis. N. Engl. J. Med. **334**:209–215.
24. **Groves, M. G., G. L. Dennis, H. L. Amyx, and D. L. Huxsoll.** 1975. Transmission of *Ehrlichia canis* to dogs by ticks (*Rhipicephalus sanguineus*). Am. J. Vet. Res. **36**:937–940.
25. **Hopp, T. P., and K. R. Woods.** 1981. Prediction of protein antigenic determinants from amino acid sequences. Proc. Natl. Acad. Sci. USA **78**:3824–3828.
26. **Hueck, C. J.** 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol. Mol. Biol. Rev. **62**:379–433.
27. **Johnson, T. C., R. B. Thompson, and T. O. Baldwin.** 1986. Nucleotide sequence of the *luxB* gene of *Vibrio harveyi* and the complete amino acid sequence of the β subunit of bacterial luciferase. J. Biol. Chem. **261**:4805–4811.
28. **Keefe, T. J., C. J. Holland, P. E. Salyer, and M. Ristic.** 1982. Distribution of *Ehrlichia canis* among military working dogs in the world and selected civilian dogs in the United States. J. Am. Vet. Med. Assoc. **181**:236–238.
29. **Kim, H. Y., and Y. Rikihisa.** 1998. Characterization of monoclonal antibodies to the 44-kilodalton major outer membrane protein of the human granulocytic ehrlichiosis agent. J. Clin. Microbiol. **36**:3278–3284.
30. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157**:105–132.
31. **Maeda, K., N. Markowitz, R. C. Hawley, M. Ristic, D. Cox, and J. E. McDade.** 1987. Human infection with *Ehrlichia canis*, a leukocytic rickettsia. N. Engl. J. Med. **316**:853–856.
32. **Mulligan, M. E., D. K. Hawley, R. Entriken, and W. R. McClume.** 1984. Analysis of the occurrence of promoter-sites in DNA. Nucleic Acids Res. **12**:789–800.
33. **Ristic, M., and C. J. Holland.** 1993. Canine ehrlichiosis. Pergamon Press. Oxford, United Kingdom.
34. **Rockey, D. D., D. Grosenbach, D. E. Hruby, M. G. Peacock, R. A. Heinzen, and T. Hackstadt.** 1997. *Chlamydia psittaci* IncA is phosphorylated by the host cell and is exposed on the cytoplasmic face of the developing inclusion. Mol. Microbiol. **24**:217–228.
35. **Rockey, D. D., R. A. Heinzen, and T. Hackstadt.** 1995. Cloning and characterization of a *Chlamydia psittaci* gene coding for a protein localized in the inclusion membrane of infected cells. Mol. Microbiol. **15**:617–626.
36. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
37. **Scidmore-Carlson, M. A., E. I. Shaw, C. A. Dooley, E. R. Fischer, and T. Hackstadt.** 1999. Identification and characterization of a *Chlamydia trachomatis* early operon encoding four novel inclusion membrane proteins. Mol. Microbiol. **33**:753–765.
38. **Stewart, G. S., S. Lubinsky-Mink, C. G. Jackson, A. Cassel, and J. Kuhn.** 1986. pHG165: a pBR322 copy number derivative of pUC8 for cloning and expression. Plasmid **15**:172–181.
39. **Sumner, J. W., W. L. Nicholson, and R. F. Massung.** 1997. PCR amplification and comparison of nucleotide sequences from the *groESL* heat shock operon of *Ehrlichia* species. J. Clin. Microbiol. **35**:2087–2092.
40. **Walker, D. H., and J. S. Dumler.** 1996. Emergence of the ehrlichioses as human health problems. Emerg. Infect. Dis. **2**:18–29.
41. **Weisiger, R. M., M. Ristic, and D. L. Huxsoll.** 1975. Kinetics of antibody response to *Ehrlichia canis* assayed by the indirect fluorescent antibody method. Am. J. Vet. Res. **36**:689–694.
42. **Weiss, E., G. A. Dasch, Y. H. Kang, and H. N. Westfall.** 1988. Substrate utilization by *Ehrlichia sennetsu* and *Ehrlichia risticii* separated from host constituents by renografin gradient centrifugation. J. Bacteriol. **170**:5012–5017.
43. **Weiss, E., J. C. Williams, G. A. Dasch, and Y. H. Kang.** 1989. Energy metabolism of monocytic *Ehrlichia*. Proc. Natl. Acad. Sci. USA **86**:1674–1678.
44. **Wells, M. Y., and Y. Rikihisa.** 1988. Lack of lysosomal fusion with phagosomes containing *Ehrlichia risticii* in P388D1 cells: abrogation of inhibition with oxytetracycline. Infect. Immun. **56**:3209–3215.

Editor: J. T. Barbieri

DNA Sequence, 2003 Vol. 14 (2), pp. 109–121

Appl. No. 10/004,494
Declaration Of Yung-Fu Chang, Ph.D. Under 37 C.F.R. §1.132
Exhibit 4

Full Length Research Paper

Cloning and Characterization of Putative Zinc Protease Genes of *Ehrlichia canis*

CHING-HAO TENG[a], STEPHEN C. BARR[b] and YUNG-FU CHANG[a,*]

[a]*Department of Population Medicine and Diagnostic Sciences;* [b]*Department of Clinical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, USA*

(Received 1 October 2002)

A putative zinc protease gene operon from *Ehrlichia canis* was cloned and sequenced. A genomic library was constructed in a pHG165 plasmid vector using *Sau*3A partially digested *E. canis* chromosomal DNA. Sequence analysis of the insert DNA from this clone indicated two open reading frames with a size of 1314 and 1350 bp that encodes for ProA and ProB, respectively. Based on BLAST analyses, ProA and ProB share 20–30% identities with members of the eukaryotic mitochondrial processing peptidase (MPP) subfamily, which are heterodimers containing α and β subunits. The subunits share a 20% of identity, but only MPP-β contains a conserved zinc-binding motif, His–Xaa–Xaa–Glu–His (HXXEH). *proA* and *proB* are also detectable in *E. canis* and *Ehrlichia chaffeensis*, but not the *Anaplasma phagocytophila*. 5′-RACE revealed that the 5′ end of the *proA* mRNA is heterogeneous, containing additional adenine residues that may be directed by pseudo-templated transcription. Although ProA was identified in both *E. canis* and *E. chaffeensis*, ProB was detected only in *E. canis*. ProA and ProB were both detectable in *E. canis*-infected DH82 cells. Sera from dogs, which were either naturally or experimentally infected with *E. canis*, recognized both the recombinant protein antigens.

Keywords: *Ehrlichia canis*; Zinc protease; Transcriptional slippage; ProA; ProB

INTRODUCTION

Canine monocytic ehrlichiosis (CME) is a potentially fatal tick-borne disease caused by the rickettsia *Ehrlichia canis* (Ristic and Holland, 1993). *E. canis* is an obligate intracytoplasmic rickettsia and replicates primarily in monocytes and lymphocytes (Ristic and Holland, 1993). The recent emergence of *Ehrlichia chaffeensis* and *Anaplasma phagocytophila* that infect human patients together with the recent report of human infection with *Ehrlichia ewingii* (Buller *et al.*, 1999) clearly indicates that it is pivotal to identify and characterize the virulent factors from these organisms.

The pitrilysin (M16) family of metallopeptidases, whose members contain a zinc-binding motif (the catalytic site) His–Xaa–Xaa–Glu–His (HXXEH), are divided into two subfamilies (Rawling and Barrett, 1995). One subfamily includes the *Escherichia coli* periplasmic oligopeptidase pitrilysin, and the *pqqF* gene product from *Klebsiella pneumoniae*. The other subfamily includes the eukaryotic mitochondrial processing peptidase (MPP), which is a heterodimer containing α and β subunits. The subunits share a high degree of identity, but only the MPP-β has a zinc-binding motif (Rawling and Barrett, 1995). Bacteria also encode MPP-like proteins, such as PqqF and PqqG (formerly PqqE and PqqF, respectively) from *Methylobacterium extrorquens* AM1 (Springer *et al.*, 1996). PqqF contains a catalytic zinc-binding motif and is an analog of MPP-β. PqqG, which shows some identity to the C-terminal domain of PqqF and both units of the MPP subfamily, is believed to be an analog of MPP-α because it lacks catalytic residues (Springer *et al.*, 1996). Although PqqF in *M. extrorquens* and PqqF in *K. pneumoniae* belong to different subfamilies, *pqqF* from *K. pneumoniae* can partially suppress the effect of

*Corresponding author. Tel.: +1-607-253-3675. Fax: +1-607-253-3943. E-mail: yc42@cornell.edu

ISSN 1042-5179 print/ISSN 1029-2365 online © 2003 Taylor & Francis Ltd
DOI: 10.1080/1042517031000073736

a *pqqF* mutation in *M. extrorquens* (Springer *et al.*, 1996). PqqFG from *M. extrorquens* and PqqF from *K. pneumoniae* are all required for synthesis of pyrroloquinoline quinone (PQQ), a prosthetic group required by quinoproteins (Duine *et al.*, 1987). Several quinoproteins, including methanol dehydrogenase (MDH) from methylotrophic bacteria and glucose dehydrogenase (GDH) from *Acinetobacter calcoaceticus*, have been well studied (Duine *et al.*, 1987). However, no published data are available for *E. canis*.

In order to identify potential ehrlichial antigens, an *E. canis* genomic library was constructed and screened with pooled dog convalescent sera. Several positive clones have been identified. One of the clones harbors a DNA fragment that contains a possible operon of two open reading frames (*proA* and *proB*). The putative proteins encoded by these two genes show extensive identities to the pitrilysin and MPP metallopeptidase subfamilies. Metallopeptidases in the MPP subfamily are composed of two similar subunits, and one of the subunits usually contains a conserved zinc-binding motif. Like other MPP subfamily members, ProA and ProB share some identity, and ProA contains a conserved zinc-binding motif. The *proA* transcription start site was also identified. The 5′ end of the *proA* transcript was found to contain various numbers of additional adenine residues that may be directed by pseudo-templated transcription (Jacques and Kolakofsky, 1991).

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The *E. canis* Oklahoma strain, *E. chaffeensis* (ATCC# CRL-10679) were cultivated in DH82 dog macrophage and *A. phagocytophila* (WI-1 strain) was cultivated in HL60 cell lines, as previously described (Dawson *et al.*, 1991; 1993; Goodman *et al.*, 1996; Dumler *et al.*, 2001). Bacteria were counted by microscopy and infection rates were determined by Diff–Quick staining. *E. coli* DH5α (F− Φ 80d *lacZ*ΔM15 (*lacZYA-argF*) U169 *endA1 recA1 hsdR17*(rk− mk+) *deoR thi-1 phoA supE44*λ-*gyrA96 relA1*)) were used for plasmid pHG165 (Stewart *et al.*, 1986) and pRSETB (Invitrogen, CA) manipulations. *E. coli* TB1 and *E. coli* BL21 (DE3) pLysS (F−, *ompT*, *hsdS*$_B$, (r_B−,m_B−), *dcm*, *gal*, λ (DE3), Cm^r) served as hosts for the *E. canis* genomic library and plasmids that express of recombinant ProA (rProA) and recombinant ProB (rProB), respectively. *E. canis* organisms were purified by the Renografin gradient centrifugation procedure as described previously (Weiss *et al.*, 1988; 1989).

Library Construction and Screening

E. canis genomic DNA was extracted from purified organisms as previously described (Chang *et al.*, 1987). Purified DNA was subjected to *Sau*3A partial digestion, and the 3–8 kb fragments were isolated and ligated to *Bam*HI digested plasmid pHG165 (Stewart *et al.*, 1986). The resulting plasmids were transferred to *E. coli* TB1 and recombinants were screened with dog anti-*E. canis* antisera as previously described (Chang *et al.*, 1987). The anti-*E. canis* antisera were prepared from beagles inoculated with *E. canis*-infected DH82 cells by IV injection. The infection was confirmed by detection of *E. canis* morulae in peripheral blood monocytes. The antiserum was preabsorbed with boiled *E. coli* TB1 lysate for 1 h at 37°C before use.

Southern Blot Analysis

E. canis genomic DNA was prepared as previously described, digested with *Hin*dIII, and separated on 0.7% agarose gel. The DNA was then transferred to nitrocellulose membranes, and probed with DNA fragments derived from the *proA* or *proB* genes (designated as Probe A and Probe B, respectively). The 1364 bp Probe A was amplified by PCR of *proA* using the primers 4RR (5′-CATGCACATGGTATTA-TACAAAGTC-3′) and ProAR (5′-TCATAAGCGTAT-CCTGCT-3′). The 747 bp Probe B was amplified by PCR of *proB* using the primers ProBF1 (5′-ATGAG-AAACATATTGTGTTAC-3′) and CH2RF (5′-TAAC-AGTCGTATCTGGTATGGTAT-3′). pCH4 served as the template in both PCR reactions. The probes were labeled with a non-radioactive labeling kit (ECL™ direct nucleic acid labeling and detection systems, Amersham, Little Chalfont, Buckinghamshire, England). Southern blot hybridization and detection were performed as described by the manufacturer.

5′-Rapid Amplification of *c*DNA Ends (5′-RACE)

To determine the transcription site of *proA*, the 5′-RACE procedure was followed with modifications (Frohman, 1993). Briefly, RNA was extracted from *E. canis* infected DH82 cells using RNAzol (Tel-test, Friendswood, TX) and the cDNA (anti-sense) was synthesized using the *proA*-specific primer ARACE1 (5′-TGCTTGGCTTTCAAC-TCTCA-3′). RNA was then degraded with RNase H (Gibco-BRL, Grand Island, NY) and cDNA was purified with the PRC purification Kit (Gibco-BRL). After an oligo-dC anchor sequence was added to the 3′ end of the cDNA using TdT and dCTP, PCR amplification was accomplished with two primers: a deoxynosine-containing anchor primer,

Qc (5′-CCAGTGAGCAGAGTGACGAGGACTCGA-GCTCAAGCTTGGGIIGGGIIGGGIIG-3′), which anneals to the poly-C tail of the cDNA and a nested *proA*-specific primer, 2RACE2 (5′-TTAATGCTTTGT-CGGTAACCT-3′). A second round of nested PCR was then performed with primer Qo (5′-CCAGTG-AGCAGAGTGACG-3′), which was complementary to the 5′ end of Qc, and the *proA*-specific primer, ARACE3 (5′-TCTGCATTCTGTCTGATTCAA-3′). The resulting PCR product was cloned into a TA cloning vector (Invitrogen, CA) and subjected to DNA sequencing. 5′ RACE was repeated four times and three clones from each experiment were subjected to DNA sequencing.

PCR Amplification and DNA Manipulations

All standard DNA manipulations and analyses, except where mentioned, were performed according to the procedures described by Sambrock *et al.* (1989). pTPROA and pTPROB, which express the rProA and rProB proteins, respectively, were constructed by cloning *proA* or *proB* sequences into pRSET (Invitrogen) in frame with the vector-encoded N-terminal 6 × His and Xpress epitope. rProA lacks the first 20 amino acids of ProA, and rProB lacks the first 94 amino acids of ProB. *proA* or *proB* sequences were amplified from pCH4 DNA by PCR using the primers NproAF (5′-CGGAATTCGCTTTATCCTTTAACATT-3′) and ProAR (5′-GCAAGCTTCATAAGCGTATCC-TGCT-3′) or ProBF2 (5′-CAGGATCCGAAGGCAAA-GGTATAGA-3′) and ProBR (5,-CCCTGCAGTCCGG-TAGAGCTAACACT-3′), respectively. PCR was carried out in a Perkin–Elmer Gene Amp PCR system 9600 thermal cycler. The amplification reaction was performed in a final volume of 50 μl containing 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.4 mM (each) deoxynucleoside triphosphate (Pharmacia), primers (0.2 μM), 1.25 U of Taq polymerase (GIBCO BRL), and 1 μl of template DNA. After the template was denatured at 94°C for 30 s, 35 amplification cycles consisting of 30 s of denaturation at 94°C, 45 s of annealing at 45°C, and 30 s of primer extension at 72°C were performed, followed by an extension cycle of 72°C for 15 min. The PCR product of partial *proA* gene was cut with *Eco* RI and *Hind*III (Invitrogen, CA) and ligated to pRSETB. The PCR product of partial *proB* fragment was digested with *Pst*I and *Bam*HI (Invitrogen, CA) and ligated into pRSETA. Plasmids were then transferred into *E. coli* strain BL21 (DE3) pLysS.

pPROAB, which was used for complementation analysis in *Methylobacterium extorquens* AM1, was created in two steps. First, a *Dra* I fragment from pCH4 containing the *proAB* genes and the *proA* promoter region was ligated to a *Sma* I-digested pUC19 plasmid. This plasmid was then digested with *Pst* I and *Bam* HI, and the fragment containing *proAB* was cloned into pRK310 (Tc^r IncP1) (Springer *et al.*, 1996).

Expression and Partial Purification of Recombinant Proteins (rProA and rProB) and Antiserum Production

E. coli BL21 (DE3) pLysS strains harboring pTPROA or pTPROB were grown in LB medium to OD_{600} = 0.5. IPTG was added to a final concentration of 1 mM and the culture was grown for 3 h. Cells expressing rProA were harvested by centrifugation at 10,000 × g for 5 min, resuspended in 6 M guanidinium (pH 7.8), and passed through a French press at 6000 psi. After rProA was partially purified by immobilized metal affinity chromatography through its N-terminal 6 × His tag in urea buffer, the purified sample was dialyzed with PBS to remove the urea. Cells expressing rProB formed insoluble inclusion bodies. The cells were harvested by centrifugation as described above, resuspended in PBS, and ruptured with a French Pressure Cell Press (American Instrumental Company, Silver Spring, MA) at 8000 psi. The total lysate was then centrifuged at 12,000 × g for 5 min at 4°C, and the resulting pellet was washed twice in nine volumes (v/v) PBS containing 0.5% TritonX-100 and 10 mM EDTA. The final product was resuspended in SDS sample buffer and boiled for 5 min to dissolve the inclusion bodies.

For antiserum production, partially purified protein samples were further separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After staining with Coomassie blue, the desired protein bands were excised, ground and mixed with equal amounts of PBS. Polyclonal antisera against rProA and rProB were raised in New Zealand White rabbits as previously described (Chang *et al.*, 1989).

SDS-PAGE and Western Blot Analysis

SDS-PAGE and Western blot analysis was performed as previously described (Chang *et al.*, 1993; 1995). To detect rProA and rProB expression, the primary antibody, MAb against the Xpress™ epitope (Invitrogen, CA), and the secondary antibody, alkaline phosphatase-conjugated affinity-purified anti-mouse IgG (KPL), were used at a dilution of 1:5000. To investigate the expression of ProA and ProB in *Ehrlichia* spp., rabbit anti-rProA and -rProB sera were used at a 1:500 dilution, and the secondary antibody, goat alkaline phosphatase-conjugated anti-rabbit IgG (KPL), was used at a 1:5000 dilution. To determine whether naturally or experimentally infected dog sera contain antibodies against ProA or ProB, test sera were used at a 1:500 dilution, and the secondary antibody, goat anti-dog IgG conjugated to alkaline phosphatase (KPL), was used at a 1:5000 dilution.

Cellular Fractionation

Ehrlichial organisms that were harvested from 39,000 cm^2 of 80% *E. canis*-infected DH82 cell culture were purified as described previously (Weiss *et al.*, 1989) and resuspended in 1 ml of SPK buffer (0.2 M sucrose and 0.05 M potassium phosphate buffer, pH 7.4) (Weiss *et al.*, 1989). Purified organisms were then sonicated at scale 4 for 2 min on ice (Sonifier Model W-185, Branson Sonic Power Company, Danbury, CT). Unbroken cells were removed by centrifugation at 1000 × g for 10 min at 4°C and the supernatant was ultracentrifuged for 1 h at 100,000 × g at 4°C. The resulting supernatant contained soluble proteins (cytoplasmic and periplasmic proteins) and the pellet (crude membranes) contained inner- and outer-membrane fractions. The soluble protein fraction was concentrated by precipitation with 5% (w/v) TCA. To separate inner and outer membranes, the method described by Ohashi *et al.* (1998) was followed with some modifications. Briefly, crude membranes were incubated in 1.5% sarkosyl solution at 37°C for 30 min and then subjected to centrifugation at 10,000 × g for 30 min at 4°C. The supernatant, which contains inner membrane proteins, was then collected. The pellet, which contains outer membrane proteins, was washed once with 2% sarkosyl buffer.

Complementation Analysis

M. extorquens AM1 *pqqF* and *pqqG* mutants were grown on minimal medium containing 0.2% succinate at 30°C as described previously (Springer *et al.*, 1996). To transfer pProAB into *M. extorquens* AM1 mutant strains, triparental matings were performed using the following strains: *E. coli* HB101 harboring the mobilizing plasmid pRK2013 (Km^r), *E. coli* harboring pProAB (Tc^r) and *M. extorquens* AM1 *pqqF* or *pqqG* mutants (Rif^r) (Springer *et al.*, 1996). The donor, mobilizer and recipient strains were mixed in a 1:1:5 ratio and spread onto a nitrocellulose membrane. After the membrane was incubated on nutrient agar medium at 30°C for 18 h, the bacteria were washed from the membrane and plated onto nutrient agar medium containing 25 μg/ml rifampicin and 12.5 μg/ml tetracycline to select for the pPROAB-harboring *M. extorquens*. The transconjugants were then plated onto minimal medium plates containing 0.5% methanol.

Nucleotide Sequence Accession Number

The DNA sequence of *proAB* has been submitted to Genbank and assigned number AF219120.

RESULTS

Sequence Analysis of *ProAB* and Surrounding Region

To identify *E. canis* proteins that are recognized by the canine humoral immune response during infection, a plasmid library of *E. canis* genomic DNA was screened with polyclonal antisera prepared from infected dogs. One clone harboring plasmid pCH4 reacted with the dog anti-*E. canis* sera.

The *E. canis* DNA insert in pCH4 was subjected to DNA sequence analysis. The 5299 bp insert contains four intact open reading frames and one partial open reading frame (Fig. 1). The first open reading frame, *ec3*, encodes a putative product of 175 amino acids possessing significant identity to the cytochrome C assembly protein. The second ORF, named *proA*, begins 402 bp downstream from the end of *ec3* and encodes a polypeptide of 438 amino acids. The putative ProA protein shares extensive identity with the pitrilysin family, which belongs to the group of metallopeptidases. The identity is highest in the N-terminal portion, which includes the conserved putative metal binding sequence, HXXGH as well as a conserved Glu for catalysis (Fig. 2). BLAST analysis reveals that ProA shares significant identity to MPP-β peptidase in *Solanum tuberosum* (27% over 284 amino acids; Genbank accession number X80237), *E. coli* pitrilysin (27% over 222 amino acids; Genbank accession number M17095), and many bacterial putative zinc proteases such as: PqqF in *M. extrorquens* AM1 (36% over 376 amino acids; Genbank accession number L43135), Y4wA in *Rhizobium* sp. (34% over 416 amino acids; Genbank accession number AE000103), putative gene product of PA0372 in *Pseudomonas aeruginosa* (27% over 419 amino acids; Genbank accession number AE004475) and, the putative gene product of HP1012 in *Helicobacter pylori*, strain 26695 (26% over 389 amino acids; Genbank accession number AE000609).

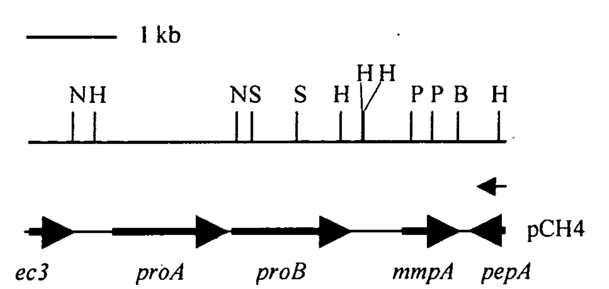

FIGURE 1 The ehrlichial DNA insert in pCH4 with their restriction map. The location and direction of the genes are indicated by bold arrows. The direction of the *lac* promoter from plasmid pHG165 is indicated by a thin arrow. Abbreviation: B, *Bam*HI; H, *Hin*dIII; N, *Nde*I; P, *Pst*I; and S, *Sau*3A. *ec3*, a putative cytochrome C assembly protein gene; *mmpA*, a putative morula membrane protein A; *pepA*, a putative lipoprotein peptidase.

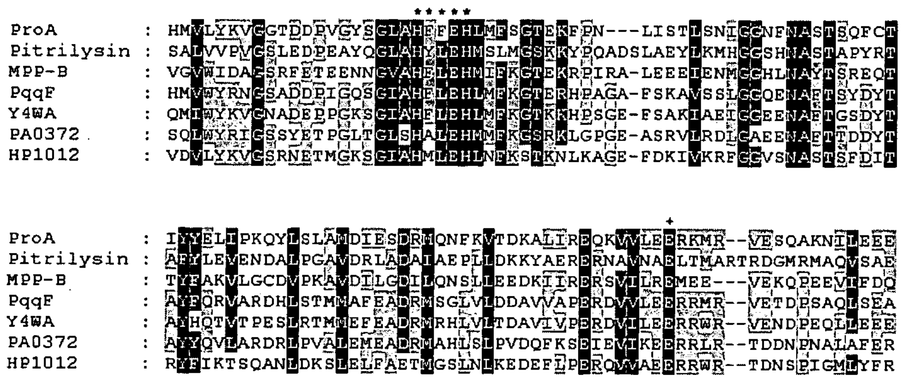

FIGURE 2 Alignment of ProA with members of the pitrilysin family and bacterial putative zinc proteases around the zinc-binding motif (HXXEH), which is indicated as "*", and the conserved catalytic Glu residue, which is indicated by " + ". Pitrilysin, *E. coli* pitrilysin; MPP-β, *S. tuberosum* (potato) MPP β subnit; PqqF, *M. extrorquens* AM1 PqqF; Y4WA, PA0372 and HP1021, putative zinc proteases from *Rhizobium* sp., *P. aeruginosa* and *H. pylori*, respectively.

The third ORF, *proB*, begins 2 bp after the end of *proA* and encodes a protein of 451 amino acids. Although without the conserved zinc-binding domain, based on the BLAST analysis ProB showed extensive identity to both subunits of MPP subfamily and some of the bacterial putative zinc proteases such as the PqqF, Y4wA and the gene products of PA0372. There are several bacterial putative proteases, which contain no zinc-ligand motif but show similarities with the pitrilysin family, have identities with ProB: PqqG, Y4wB, gene product of PA0371 and HP0657 in *M. extrorquens* AM1, *Rhizobium* spp., *P. aeruginosa* and *H. pylori* 26695, respectively. In addition, ProA and ProB showed 20% of identity (over 227 amino acid) on their N-terminal ends. The fourth ORF, *mmpA*, encodes a polypeptide of 174 amino acids that does not have significant identity to any proteins in the databases. The fifth partial ORF encodes a putative lipoprotein signal peptidase.

Using the homology score method (Mulligan *et al.*, 1984), consensus −10 (TAAACT) and −35 (TTGATA) δ^{70} binding sites were found upstream of *proA* (Fig. 3). In addition, potential ribosome-binding sites (AAGGT and GGAGA) were found upstream from the translation initiation codons of *proA* and *proB*, respectively (Fig. 3). A potential transcription terminator was also identified downstream of *proB* (Fig. 3). Based on the method developed by Heijne, both ProA and ProB contain a N-terminal signal sequence (von Heijne, 1986).

Identification of *proA* and *proB* Homologous Genes in Other *Ehrlichia* Strains

To investigate whether *proA* and *proB* homologs exist in other *Ehrlichia* strains, Southern blot analysis was performed on *Hin*dIII-digested genomic DNA from *E. chaffeensis* and *A. phagocytophila* (Fig. 4). Because ehrlichiae must be grown in host cells, the genomic DNA preparations also contain host cell DNA. Thus, pure host cell genomic DNAs (from DH82 and HL60 cells) were also prepared to serve as negative controls. pCH4 was used as a positive control. *proA* and *proB* sequences were detected using PCR-amplified probes designated probe A and B, respectively. Both probe A and B hybridize to a 2.7 kb fragment in pCH4 and *E. canis*-infected DH82 cell DNA (Fig. 4, lanes 1 and 3), as well as a 2.5 kb fragment in *E. chaffeensis*-infected DH82 cell DNA (Fig. 4, lane 4), but negative in DH82 cell DNA (Fig. 4, lane 2). Probe A and B did not hybridize to DNA prepared from *A. phagocytophila*-infected HL60 or HL60 cells (Fig. 4, lanes 5 and 6). These results suggest that both *E. canis* and *E. chaffeensis* contain a single copy of the *proA* and *proB* genes.

5′-RACE for Determination of the Transcript Start Site of *proAB* Operon

To determine the transcriptional start site of *proA*, 5′-*R*apid *A*mplification of *c*DNA *E*nds (5′-RACE) was performed as described in the "Materials and methods" section. Briefly, a poly-C oligonucleotide sequence was appended onto the 3′ end of the cDNA (template strand) synthesized from *proA* mRNA. The cDNA product was then amplified by PCR using the poly-C as the anchor sequence, cloned into a TA cloning vector, and sequenced. The transcription start site of *proA* can be ascertained by determining the point at which the poly-G sequence (poly-C in the template strand of the cloned PCR product) was appended onto the 5′ end of the *proA* cDNA (coding strand). However, this experiment was repeated four times, and a variable number of As (4, 5, 6, or 17) not found in the *proA* DNA sequence were detected at the 5′ end of the *proA* 5′-RACE PCR product following the poly-G sequence (Fig. 5). These results might be explained by a phenomenon called pseudo-templated transcription, which has been documented in both prokaryotic and eukaryotic organisms (Jacques and Kolakofsky, 1991).

Excluding the additional variable poly-A sequence, the 5′ end of the mRNA is an AAA sequence located 14–16 bp upstream of the *proA* translation start site (Fig. 5).

Expression and Purification of rProA and rProB

To express ProA and ProB in *E. coli*, recombinant truncated versions of these proteins were constructed that contain N-terminal 44-amino acid sequence tags, which include a 6 × His Tag and a Xpress™ epitope. The recombinant genes expressing these proteins were constructed by PCR-amplifying *proA* and *proB*, and cloning the products into the expression vector pRSET. The resulting plasmids, pTPROA and pTPROB were transferred into *E. coli* BL21, and the recombinant proteins, rProA and rProB were expressed and partially purified as described in the "Materials and Methods" section. rProA and rProB expression was detected by

```
                                                  TTTACTCTTATTTTTTTAT
 -60 CACTTGATATTATTAAATAATCATATAAACTCCCAAATAAACTATTGCAAGGTTATGGTA
        -35                   -10          *
   1 ATGATGAAATTTTTTACTTGTTTTTTCATAGTTTTCTTAACAATAGCCAATCATGCTTTA
     M  M  K  F  F  T  C  F  F  I  V  F  L  T  I  A  N  H  A  L
  61 TCCTTTAACATTAAAGTTACACATGAAAAATTAGATAATGGAATGGAAGTATACGTGATT
     S  F  N  I  K  V  T  H  E  K  L  D  N  G  M  E  V  Y  V  I
 121 CCAAATCATCGCGCACCAGCAGTCATGCACATGGTATTATACAAAGTCGGTGGAACTGAT
     P  N  H  R  A  P  A  V  M  H  M  V  L  Y  K  V  G  G  T  D
 181 GATCCAGTAGGATACTCTGGATTAGCACATTTTTTTGAACACTTAATGTTTAGTGGAACA
     D  P  V  G  Y  S  G  L  A  H  F  F  E  H  L  M  F  S  G  T
 241 GAAAAATTTCCTAATCTCATCAGCACACTTAGTAATATAGGCGGAAATTTCAATGCAAGC
     E  K  F  P  N  L  I  S  T  L  S  N  I  G  G  N  F  N  A  S
 301 ACATCTCAATTTTGTACTATATACTACGAATTAATACCAAAACAATATTTATCTCTTGCA
     T  S  Q  F  C  T  I  Y  Y  E  L  I  P  K  Q  Y  L  S  L  A
 361 ATGGATATTGAATCAGACAGAATGCAGAATTTTAAGGTTACCGACAAAGCATTAATAAGA
     M  D  I  E  S  D  R  M  Q  N  F  K  V  T  D  K  A  L  I  R
 421 GAACAAAAGGTAGTCTTAGAAGAAAGAAAAATGAGAGTTGAAAGCCAAGCAAAAAACATA
     E  Q  K  V  V  L  E  E  R  K  M  R  V  E  S  Q  A  K  N  I
 481 CTAGAAGAAGAAATGGAAAATGCATTTTATTACAATGGATATGGCAGACCAGTAGTAGGA
     L  E  E  E  M  E  N  A  F  Y  Y  N  G  Y  G  R  P  V  V  G
 541 TGGGAACATGAAATTAGCAACTACAACAAAGAAGTTGCTGAAGCCTTTCATAAGCTACAT
     W  E  H  E  I  S  N  Y  N  K  E  V  A  E  A  F  H  K  L  H
 601 TATAGTCCTAATAATGCTATATTAATTGTAACTGGAGATGCAGATCCACAAGAAGTAATC
     Y  S  P  N  N  A  I  L  I  V  T  G  D  A  D  P  Q  E  V  I
 661 ACACTTGCAAAACAATACTATGGGAAAATACCATCTAATAATAAGAAACCTTCAAGTCAA
     T  L  A  K  Q  Y  Y  G  K  I  P  S  N  N  K  K  P  S  S  Q
 721 GTTAGGGTAGAACCACCGCATAAAACAAATATGACTTTAACATTAAAAGACAGTTCAGTA
     V  R  V  E  P  P  H  K  T  N  M  T  L  T  L  K  D  S  S  V
 781 GAAATCCCAGAACTGTTTTTAATGTATCAAATACCAAATGGTATTACCAATAAAAACTAC
     E  I  P  E  L  F  L  M  Y  Q  I  P  N  G  I  T  N  K  N  Y
 841 ATACTTAACATGATGTTAGCAGAAATACTCGGTAGTGGTAAATTCAGCCTGCTTTACAAT
     I  L  N  M  M  L  A  E  I  L  G  S  G  K  F  S  L  L  Y  N
 901 GATTTGGTAATTAACAATCCAATAGTTACATCGATAAAAACAGATTATAATTACTTAACT
     D  L  V  I  N  N  P  I  V  T  S  I  K  T  D  Y  N  Y  L  T
 961 GACAGCGATAATTACCTTTCCATTGAAGCTATACCTAAAAACGGGATCTCTACAGAAGCT
     D  S  D  N  Y  L  S  I  E  A  I  P  K  N  G  I  S  T  E  A
1021 GTAGAACAAGAAATTCATAAATGTATAAATAATTATTTAGAAAATGGAATTTCAGCAGAA
     V  E  Q  E  I  H  K  C  I  N  N  Y  L  E  N  G  I  S  A  E
1081 TATTTAGAAAGTGCAAAGTATAAAGTAAAAGCACATTTAACTTATGCATTTGACGGACTA
     Y  L  E  S  A  K  Y  K  V  K  A  H  L  T  Y  A  F  D  G  L
1141 ACTTTCATATCATATTTTTATGGCATGCATCTAATACTAGGAGTACCGCTATCAGAAATC
     T  F  I  S  Y  F  Y  G  M  H  L  I  L  G  V  P  L  S  E  I
1201 AGTAATATTTACGATACCATAGACAAAGTAAGTATCCAAGATGTTAACTCCGCTATGGAA
     S  N  I  Y  D  T  I  D  K  V  S  I  Q  D  V  N  S  A  M  E
1261 AATATCTTTCAAAACAATATAAGATTAACCGGGCATTTATTACCTAATGGAGAATAGTTA
     N  I  F  Q  N  N  I  R  L  T  G  H  L  L  P  N  G  E  *    M
1321 TGAGAAACATATTGTGTTACACATTAATATTGATTTTCTTTTCATTCAATACATATGCAA
       R  N  I  L  C  Y  T  L  I  L  I  F  F  S  F  N  T  Y  A  N
1381 ATGATCTCAATATTAACATAAAAGAAGCTACAACTAAAAATAAAATACACTATCTATATG
       D  L  N  I  N  I  K  E  A  T  T  K  N  K  I  H  Y  L  Y  V
1441 TTGAACATCATAACCTACCAACAATTTCCTTAAAATTTGCATTCAAGAAAGCAGGATACG
       E  H  H  N  L  P  T  I  S  L  K  F  A  F  K  K  A  G  Y  A
1501 CTTATGATGCCTTTGATAAGCAAGGACTTGCATACTTTACATCAAAAATATTAAACGAAG
```

```
1561 GATCAAAAAACAACTATGCTCTCAGTTTTGCACAACAATTAGAAGGCAAAGGTATAGACT
        S  K  N  N  Y  A  L  S  F  A  Q  Q  L  E  G  K  G  I  D  L
1621 TAAAATTTGATATAGACCTAGACAATTTTTATATATCATTAAAAACCTTATCAGAAAACT
        K  F  D  I  D  L  D  N  F  Y  I  S  L  K  T  L  S  E  N  F
1681 TTGAAGAAGCCCTAGTTTTACTCAGTGATTGCATATTCAACACCGTCACAGATCAAGAAA
        E  E  A  L  V  L  L  S  D  C  I  F  N  T  V  T  D  Q  E  I
1741 TATTCAATAGAATAATAGCAGAACAGATTGCACATGTTAAATCATTATATTCTGCTCCTG
        F  N  R  I  I  A  E  Q  I  A  H  V  K  S  L  Y  S  A  P  E
1801 AATTTATAGCTACAACAGAAATGAATCACGCTATATTCAAAGGGCACCCATATTCTAACA
        F  I  A  T  T  E  M  N  H  A  I  F  K  G  H  P  Y  S  N  K
1861 AAGTTTACGGGACATTAAATACAATCAATAATATCAACCAGGAAGACGTTGCATTATATA
        V  Y  G  T  L  N  T  I  N  N  I  N  Q  E  D  V  A  L  Y  I
1921 TAAAAAATAGTTTTGACAAGGAACAAATCGTTATCAGCGCAGCAGGAGATGTAGATCCAA
        K  N  S  F  D  K  E  Q  I  V  I  S  A  A  G  D  V  D  P  T
1981 CACAGCTATCAAATTTACTAGATAAATATATTCTTTCCAAATTGCCATCTGGTAATAACA
        Q  L  S  N  L  L  D  K  Y  I  L  S  K  L  P  S  G  N  N  K
2041 AAAATACCATACCAGATACGACTGTTAATAGAGAAGACACATTATTATATGTACAGAGAG
        N  T  I  P  D  T  T  V  N  R  E  D  T  L  L  Y  V  Q  R  D
2101 ATGTACCACAAAGTGTCATAATGTTTGCTACAGACACAGTACCATATCACAGCAAAGACT
        V  P  Q  S  V  I  M  F  A  T  D  T  V  P  Y  H  S  K  D  Y
2161 ATCATGCATCAAACTTGTTCAATACTATGCTAGGCGGATTAAGTCTCAATTCAATATTAA
        H  A  S  N  L  F  N  T  M  L  G  G  L  S  L  N  S  I  L  M
2221 TGATAGAATTAAGAGACAAGTTAGGATTAACATACCATAGTAGCAGTTCACTATCTAACA
        I  E  L  R  D  K  L  G  L  T  Y  H  S  S  S  S  L  S  N  M
2281 TGAATCATAGTAATGTGCTATTTGGTACAATATTCACTGATAATACCACAGTAACAAAAT
        N  H  S  N  V  L  F  G  T  I  F  T  D  N  T  T  V  T  K  C
2341 GTATATCCGTCTTAACAGATATTATAGAGCACATTAAAAAGTATGGAGTTGATGAAGACA
        I  S  V  L  T  D  I  I  E  H  I  K  K  Y  G  V  D  E  D  T
2401 CTTTTGCAATTGCAAAATCTAGTATTACCAACTCTTTTATTTTATCTATGTTAAATAACA
        F  A  I  A  K  S  S  I  T  N  S  F  I  L  S  M  L  N  N  N
2461 ATAATGTTAGTGAGATATTGTTAAGCTTACAATTACACGATCTAGATCCGAGTTATATTA
        N  V  S  E  I  L  L  S  L  Q  L  H  D  L  D  P  S  Y  I  N
2521 ATAAATACAATTCTTACTACAAAGCAATAACAATAGAAGAAGTAAATAAAATTGCCAAGA
        K  Y  N  S  Y  Y  K  A  I  T  I  E  E  V  N  K  I  A  K  K
2581 AAATTTTATCTAATGAATTAGTAATAATTGAAGTAGGAAAAAACAATAACATAAATGGCA
        I  L  S  N  E  L  V  I  I  E  V  G  K  N  N  N  I  N  G  K
2641 AACAAATAGATGCTAAAAAACACATACTTGGTTAAGTATACAGGTTATTGTATTTACTAC
        Q  I  D  A  K  K  H  I  L  G  *
2701 AAGTATTCTATTAGGTTGTATTAAGTAAGTATAAGTAGCTTCAATCAAATAAAAAAACAT
2761 TAACCAAAGTGTTAGCTCTACCGGAGAAGCTTATTATAAGCTTTTAACCTGGGATAATAT
2821 GAAGTTTTGCTAATGTTAAGCAAAAAATTAGTAATCACAATATCAAATTTTCTTTACAGG
          ------>      <------
```

FIGURE 3 Nucleotide sequence of the putative *proAB* operon and its coding proteins (ProA and ProB). *ProA* is 2 nucleotids upstream of *proB*. Nucleotide numbers are indicated on the left. The start and stop codons are indicated in boldface type. Promoter-like region proximal to *proA* is underlined. The potential ribosome-binding sites preceding each gene are indicated underlined boldface types. A potential transcription terminator of the putative operon is indicated by ---- > < ----.

SDS-PAGE and Western blot analysis. The predicted molecular mass of rProA is 52,490 kDa, and a band of about this size is detectable when both crude and partially purified protein preparations of *E. coli*/pT-PROA are subjected to SDS-PAGE analysis (Fig. 6A). Similarly, rProB is predicted to be 44,176 kDa, and a product corresponding to this size is detectable in both crude and partially purified protein preparations of *E. coli*/pTPROB (Fig. 6A). Western blot analysis of the recombinant proteins with anti-Xpress™ monoclonal antibodies also confirmed the existence of these two proteins (Fig. 6B).

Western Blot Analysis

To determine whether ProA and ProB are expressed in *E. canis* and *E. chaffeensis*, Western blot analysis was performed on lysates of *E. canis* or *E. chaffeensis*-infected DH82 cells. ProA and ProB were detected using polyclonal antisera raised against rProA and rProB, respectively. The predicted molecular weight of native ProA is approximately 50 kDa, and anti-rProA antibodies detected proteins of similar sizes in both *E. canis* and *E. chaffeensis*-infected DH82 cells. However, the *E. chaffeensis* ProA protein appears to be slightly larger than the *E. chaffeensis* protein (Fig. 7). No specific band was detected in uninfected DH82 cells. Anti-rProB serum recognizes a 50 kDa protein from *E. canis*-infected DH82 cells, which is close to the predicted molecular weight of ProB (50.9 kDa). No specific band was detected from either *E. chaffeensis*-infected DH82 or DH82 cells.

To determine whether ProA and ProB are commonly recognized by the canine immune response

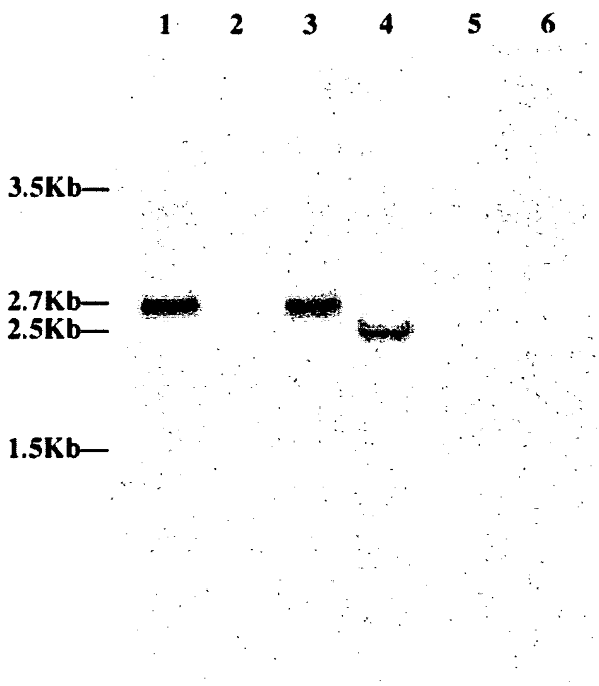

FIGURE 4 Southern blot analysis of genomic DNA digested with *Hin*dIII and hybridized with a PCR product, ProbeB, derived from *prob*. Lane 1, *Hin*dIII-digested pCH4, lanes 2, 3 and 4, genomic DNA extracted from DH82 cells, *E. canis*-infected DH82 cells, and *E. chaffeensis*-infected DH82 cells, respectively; lanes 5 and 6, Genomic DNA from HL60 cells and the HGE agent-infected HL60 cells, respectively.

during *E. canis* infection, Western blot analysis was performed on partially purified rProA and rProB preparations using antisera from three naturally infected dogs and one experimentally infected dog. Antisera from all of these infected dogs contain antibodies recognizing the two proteins. Serum from an uninfected dog or specific-pathogen-free (SPF) dog did not recognize rProA or rProB (Fig. 8).

Subcellular Localization

The location of ProA and ProB in *Ehrlichia* was determined by subcellular fractionation. As described in the "Materials and methods" section, three bacterial fractions were collected: (1) soluble cytoplasmic and periplasmic proteins, (2) inner membrane proteins and (3) outer membrane proteins. Western blot analysis of these three fractions with anti-rProA and -rProB sera reveal that both ProA and ProB are in the soluble protein fraction, although a tiny amount of ProA is detectable in the inner membrane fraction (Fig. 9). These results indicate that ProA and ProB are either cytoplasmic or periplasmic proteins.

Complementation Analyses *proAB*

ProA and ProB share significant identity with the zinc proteases PqqF and PqqG, respectively, in

5′-ACTCCCAAAT<u>AAA</u>CTATTGCAAGGTT**ATG**-3′	*proAB* **coding strand**
5′...GGGG(**A**)$_n$<u>AAA</u>CTATTGCAAGGTT**ATG**-3′ Appended poly(G)	**5′ RACE results**

FIGURE 5 5′-RACE results revealed that the putative *proAB* is transcribed from a stretch of AAA sequence (underlined) located 14–16 bp upstream of the *proA* translation start site (boldface type) and the 5′ end of its mRNA is heterogeneous with various number of additional adenines which are not seen on the corresponding region of the *proAB* coding strand. *N* is 4, 5, 6, or 17 based on four times of the 5′RACE results.

M. extorquens AM1. To determine whether ProA and ProB are functionally similar to PqqF and PqqG, plasmids containing the *proAB* region were transferred into *M. extorquens* AM1 *pqqF* or *pqqG* mutants. PqqF and PqqG are involved in the biosynthesis of PQQ, which is required for *M. extorquens* growth on methanol (Springer *et al.*, 1996). Therefore, the ability of *proA* and *proB* to restore the growth of *M. extorquens pqqF* and *pqqG* mutants on minimal medium with methanol as the sole carbon source was scored. Neither mutant harboring *proAB* was able to survive.

DISCUSSION

We have cloned and sequenced two *Ehrlichia* genes (*proAB*), which encode novel proteins (ProAB) which show identities with the metallopeptidases in the pitrylisin family. Although their enzymatic function is still unknown, these two proteins may be classified in the MPP subfamily due to the three properties that MPP members have: (1) both of these proteins share identities with MPP members, (2) only ProA has the putative catalytic domain and (3) ProAB share identities with each other (Rawling and Barrett, 1995). ProA may be the analog of β subunits of MPP, while ProB is the probable analog of α subunits of this subfamily.

Based on the BLAST analysis, ProA and ProB show high degree of identities with the PqqF and PqqG of *M. extrorquens* AM, respectively. PqqFG has been suggested to belong to the MPP subfamily with PqqF and PqqG analogous to the β and α subunits, respectively (Springer *et al.*, 1996). From the recent complete genome sequence of several bacteria (Freiberg *et al.*, 1997; Tomb *et al.*, 1997; Stover *et al.*, 2000), the gene products of *mpp*-like gene pairs were also found to share significant identities to those of *proAB*, such as product of y4wA and y4wB in *Rhizobium* sp., PA0372 and PA0371 in *P. aeruginosa* and HP0657 and HP1012 in *H. pylori*. Like ProA and PqqF, Y4wA and gene products of PA0372 and HP1012 contain the zinc-binding domain, while like ProB and PqqG, Y4wB and gene products of PA0371 and HP0615 show similarities to the MPP subfamilies and lack the zinc-binding motif.

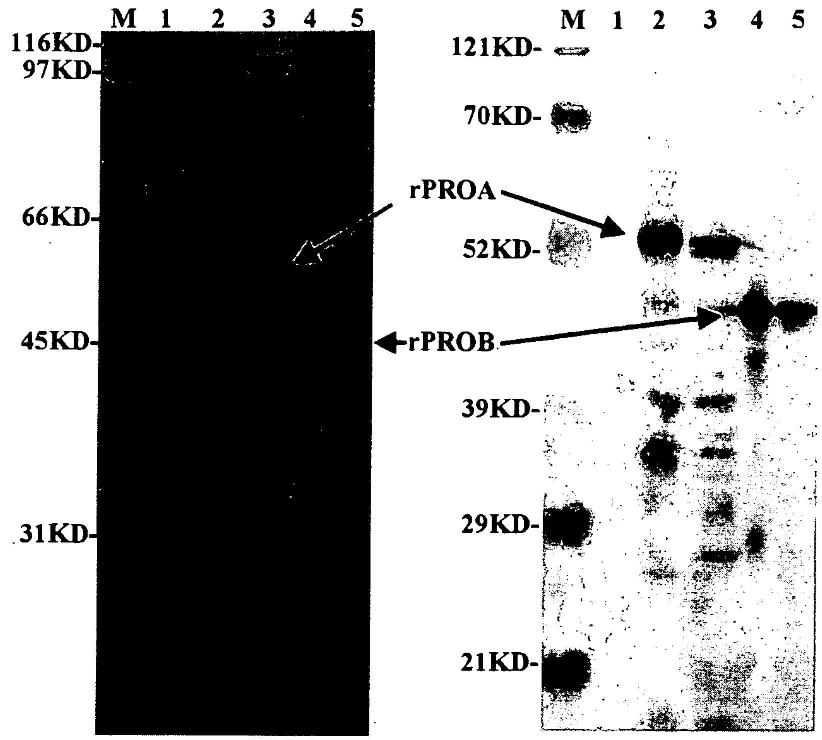

FIGURE 6 Identification of recombinant ProA and ProB (rProA and rProB, respectively) with SDS-PAGE (Panel A) and Western blotting with MAb against Xpress™ epitope (Panel B). Lane M, size marker proteins; lane 1 *E. coli* (negative control); lane 2, purified rProA; lane 3, pTPROA-transformed *E. coli*; lane 4, purified rProB; lane 5, pTPROB-transformed *E. coli*.

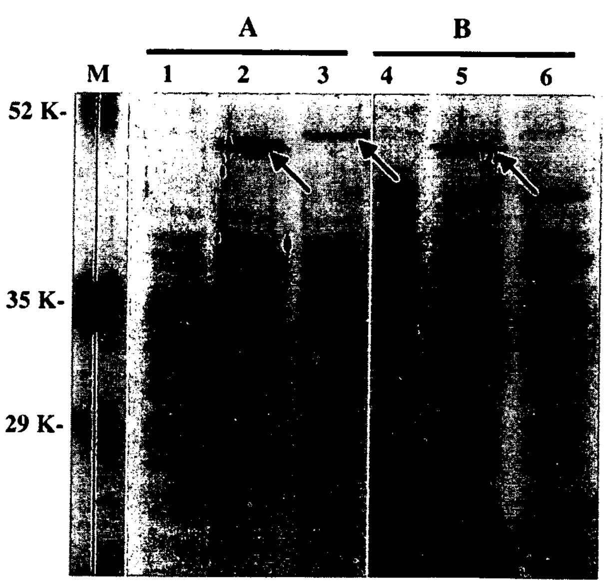

FIGURE 7 Identification of native ProA and ProB from ehrlichal organisms with Western blot assay. Samples were probed with anti-rProA (A) and anti-rProB (B) rabbit sera, respectively. Lane M protein size marker; lanes 1 and 4, DH82 cells; lanes 2 and 5; *E. canis*-infected DH82 cells; lanes 3 and 6, *E. chafeensis*-infected DH82 cells.

However, the organization of the gene pairs in the genome are different. In *E. canis*, *M. extrorquens* and *Rhizobium sp*, their MPP-like genes are colocalized with the gene coding the zinc-ligand domain on the upstream. In *P. aeruginosa*, the genes are colocalized but with the zinc-ligand containing gene on the downstream. In *H. pylori*, they are located in different loci in the genome (Freiberg *et al.*, 1997; Tomb *et al.*, 1997; Stover *et al.*, 2000).

The 5′ RACE results reveal heterogeneity at the 5′ end of the *proA* transcript resulting from the variable addition of extra A residues. This heterogeneity may be caused by pseudo-templated transcription (Jacques and Kolakofsky, 1991). Pseudo-templated transcription (also known as reiterative transcription, transcriptional slippage and RNA polymerase stuttering (Qi and Turnbough, 1995)) describes the process by which a homopolymeric stretch of nucleotides are added to a nascent RNA transcript by repetitive transcription of a shorter stretch of complementary bases (usually 3–7) in the DNA template. Pseudo-templated transcription can occur

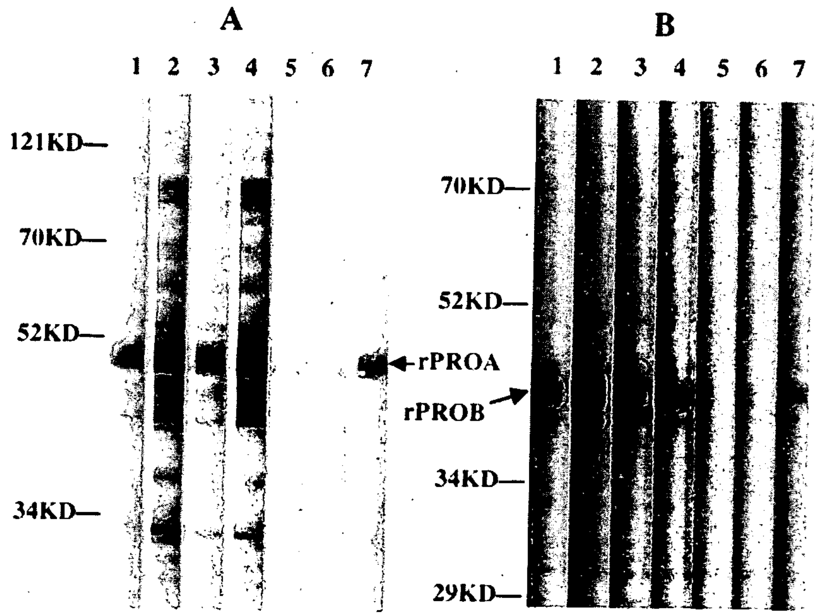

FIGURE 8 Western blot analysis of rProA (A) and rProB (B) with different canine sera. Lane 1, serum from a dog artificially infected with *E. canis* by injection of *E. canis*-infected DH82 cells; lanes 2–4, three different field *E. canis*-IFA-positive canine serum; lane 5, normal canine serum; lane 6, SPF canine serum; lane 7, the same pooled canine serum used to screen the library.

in the 5′ end, 3′ end, or internal region of otherwise normally templated RNA. This reaction has been found to occur in the mRNAs of prokaryotes, phages and eukaryotic viruses. Two essential elements are required for pseudo-templated transcription to occur: (1) a slippage site containing a short homopolymeric stretch of nucleotides (at least 3) which serve as the template for the repetitive transcription and (2) a pausing-inducing site which slows down the movement of the RNA polymerase (Jacques and Kolakofsky, 1991) and thus places the polymerase at the slippage site. If pseudo-templated transcription occurs at the 5′ end of a gene, a promoter can serve as a pausing-inducing site, since the polymerase idles at the promoter before initiation (Jacques and Kolakofsky, 1991). The *proA* promoter and the three adenine nucleotides at the transcription initiation site of *proA* (Fig. 3) fulfill the two requirements for pseudo-templated transcription. Similar cases in which transcription initiates from a stretch of As on the coding strand of DNA and results in the addition of a various number of pseudo-templated transcribed As in the 5′ end of the mRNA have been reported (Machida *et al.*, 1984; Guo and Roberts, 1990). For example, the late gene of bacteriophage 82 and the *insA* gene of insertion

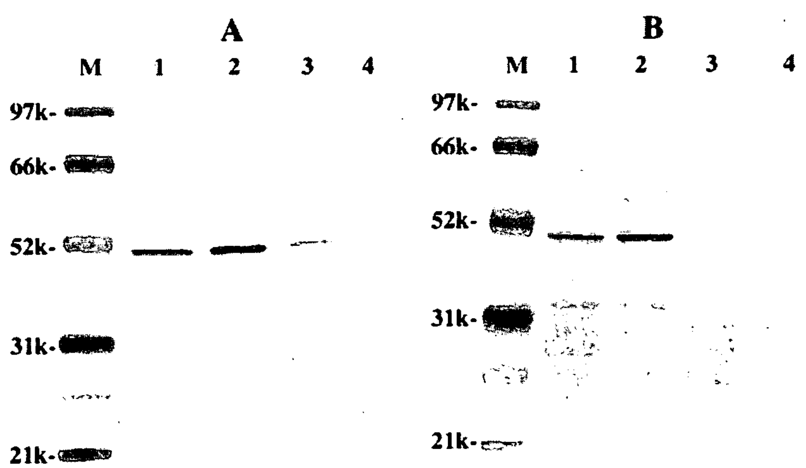

FIGURE 9 Western blot analysis of *E. canis* subcellular fractions with anti-rProA (A) and anti-ProB (B) antibodies. Lane M, protein size marker; lane 1, whole organisms of *E. canis*; lane 2, soluble protein fraction; lane 3, inner membrane fraction; lane 4, outer membrane fraction. The three subcellular fractions loaded here were adjusted to the comparable amount.

sequence IS*1* have 3 and 4 As at their transcription initiation sites, respectively (Machida *et al.*, 1984; Guo and Roberts, 1990). When transcribed by *E. coli* RNA polymerase, up to 18 or 8 pseudo-templated as were appended onto the 5′ ends of the late gene of phage 82 or *insA* transcripts, respectively. The poly (A) heads on late gene mRNAs of the Vaccinia DNA virus also result from pseudo-templated transcription of triplet A sequences at transcription initiation sites (Jacques and Kolakofsky, 1991). In some cases, pseudo-templated transcription has been shown to play a role in gene regulation. For example, several *E. coli* genes such as *pyrBI*, *codBA* and *carAB* contain a short stretch of thymine bases (at least three) close to their transcription start sites (Liu *et al.*, 1994; Qi and Turnbough, 1995; Han and Turnbough, 1998). When the UTP concentration is high, these thymines are repetitively transcribed as uridines in the nascent transcript. This process induces the release of the short transcript from the initiation complex, thus preventing the production of intact mRNA. It might also play a role in the regulation of *proAB* expression.

Southern blot analysis of *Hin*dIII-digested chromosomal DNA from *E. canis*, *E. chaffeensis* and *A. phagocytophila* with DNA probes derived from *proA* or *proB* (probe A and B, respectively) indicated that genes homologous to *proA* and *proB* are present in *E. chaffeensis*, but not in *A. phagocytophila*. This finding may be due to the different level of phylogenetic divergence among the three ehrlichiae. Based on phylogenetic analyses of 16S rRNA or *groEL* sequences in *Ehrlichia* spp., *E. canis* and *E. chaffeenis* are classified in the same genogroup, whereas *A. phagocytophila* is classified in another genogroup (Walker and Dumler, 1996; Sumner *et al.*, 1997). Probe A and B showed identical hybridization patterns to *E. canis* and *E. chaffeensis* DNA. Both probes recognized a 2.7 kb-fragment in *E. canis* and a 2.5 kb-fragment in *E. chaffeensis*. These results suggest that *proA* and *proB* are closely linked in *E. chaffeensis*, as they are in *E. canis*.

Western blot analysis of *E. canis* and *E. chaffeensis*-infected DH82 cell lysates with anti-rProA antibodies demonstrated that both *E. canis* and *E. chaffeensis* express ProA in culture. Although *proB* sequences are detectable in *E. chaffeensis* by Southern blot, Western blot analysis with anti-rProB antibodies only detects ProB expression in *E. canis* but not in *E. chaffeensis*. There are several possible explanations for this finding. First, anti-rProB antibodies may not recognize the SDS-denatured ProB homolog in *E. chaffeensis*. Second, the *E. chaffeensis* ProB homolog may be poorly expressed in DH82 cells. Finally, *proB* may be mutated in *E. chaffeensis*. Further cloning and sequencing of the *E. chaffeensis proB* gene is needed to answer this question. Based on this finding, ProB may serve as a tool for serologic differentiation of *E. canis* and *E. chaffeensis*.

This study has also shown that *E. canis* expresses ProA and ProB in dogs that are naturally infected through tick bites or artificially infected by IV inoculaton of *E. canis*-infected DH82 cells, since sera from experimentally or naturally *E. canis*-infected dogs recognized both rProA and rProB. In addition, dog anti-*E. canis* serum detected ProA and ProB in *E. canis*-infected DH82 cells in Western blot and indirect immunofluorescent assays. The antigenicity and general expression of ProAB in dog host cells suggest that these two proteins may serve as good tools for serodiagnosis of *E. canis* infection.

E. canis subcellular fractionation experiments demonstrated that ProA and ProB mainly localize in the fraction containing soluble periplasmic and cytoplasmic proteins. Analysis of the amino acid sequences of ProA and ProB reveals that these proteins might both contain N-terminal signal sequences. Thus, ProA and ProB may be periplasmic proteins like *E. coli* pitrilysin. Several attempts to precisely locate these two proteins by immunogold labelling have been failed. However, ProA and ProB appear to co-localize, suggesting that they may directly interact like other MPP-α and -β subunits, which need to cooperate to form active complexes (Rawling and Barrett, 1995).

To date, none of the enzymatic functions of the bacterial MPP-like proteins have been identified. However, based on genetic studies, the gene products of *pqqFG* in the facultative methanol-utilizing bacterium *M. extrorquens* AM1 have been identified to be required for PQQ biosynthesis (Goodwin and Anthony, 1998). PQQ is the non-covalently bound prosthetic group of many quino-protein dehydrogenases, including MDH and some GDH of Gram-negative bacteria (Neijssel, 1987). PQQ is formed by fusion of glutamate and tyrosine, but the biosynthetic pathway is unknown (Duine and Jongejan, 1989). *pqqF* and *pqqG* mutants of *M. extrorquens* lose the ability to grow on medium that contains methanol as the sole energy source. Although ProA and ProB show significant identities to PqqF and PqqG in *M. extrorquens* AM1, respectively, *proAB* failed to restore the growth of *pqqF* and *pqqG* mutants on methanol. This result may indicate that the degrees of similarities between the two pairs of proteins are not high enough to allow ProAB to functionally replace PqqFG. However, it is also possible that *proAB* are not expressed well enough in *M. extrorquens* to allow complementation.

In conclusion, we have cloned and sequenced the *E. canis proA* and *proB* genes, which encode proteins that may be new members of the MPP metallopeptidase subfamily. The 5′ end of the *proA* transcript appears to contain additional pseudo-templated A residues. Therefore, pseudo-templated transcription may play some role in the regulation of the putative *proAB* operon. The *proA* and *proB* genes

were detected in E. canis and E. chaffeensis, but not in A. phagocytophila. ProA was detected in both E. canis and E. chaffeensis, while ProB was only detected in E. canis. The finding that ProA and ProB are expressed in E. canis during both natural and experimental dog infections indicates that these proteins may be useful for serodiagnosis. Whether the *proAB* genes play a role in the pathogenesis of CME is unknown. It is impossible to study the functions of ProA and ProB *in vivo* due to the lack of genetic tools, such as allelic exchange and/or transposon mutagenesis, in *Ehrlichia*. However, further characterization of the function of these two proteins may offer significant insights into their roles in CME caused by E. canis.

Acknowledgements

This work was supported by grants from the Fort Dodge Animal Health, a Division of American Home Products Corporation and the New York State Science and Technology Foundation. We thank to Dr Lidstrom for her gifts of *Methylobacterium extorquens* AM1 *pqqE* and *pqqF* mutants, the mobilizing plasmid pRK2013, and pRK310.

References

Buller, R.S., Arens, M., Hmiel, S.P., Paddock, C.D., Sumner, J.W., Rikihisa, Y., Unver, A., Gaudreault-Keener, M., Manian, F.A., Liddell, A.M., Schmulewitz, N. and Storch, G.A. (1999) "*Ehrlichia ewingii*, a newly recognized agent of human ehrlichiosis", *N. Engl. J. Med.* **341**, 148–155.

Chang, Y.F., Young, R., Post, D. and Struck, D.K. (1987) "Identification and characterization of the *Pasteurella haemolytica* leukotoxin", *Infect. Immun.* **55**, 2348–2354.

Chang, Y.F., Young, R. and Struck, D.K. (1989) "Cloning and characterization of a hemolysin gene from *Actinobacillus (Haemophilus) pleuropneumoniae*", *DNA* **8**, 635–647.

Chang, Y.F., Shi, J., Ma, D.P., Shin, S.J. and Lein, D.H. (1993) "Molecular analysis of the *Actinobacillus pleuropneumoniae* RTX toxin-III gene cluster", *DNA Cell Biol.* **12**, 351–362.

Chang, Y.F., Appel, M.J., Jacobson, R.H., Shin, S.J., Harpending, P., Straubinger, R., Patrican, L.A., Mohammed, H. and Summers, B.A. (1995) "Recombinant OspA protects dogs against infection and disease caused by *Borrelia burgdorferi*", *Infect. Immun.* **63**, 3543–3549.

Dawson, J.E., Anderson, B.E., Fishbein, D.B., Sanchez, J.L., Goldsmith, C.S., Wilson, K.H. and Duntley, C.W. (1991) "Isolation and characterization of an *Ehrlichia* sp. from a patient diagnosed with human ehrlichiosis", *J. Clin. Microbiol.* **29**, 2741–2745.

Dawson, J.E., Candal, F.J., George, V.G. and Ades, E.W. (1993) "Human endothelial cells as an alternative to DH82 cells for isolation of *Ehrlichia chaffeensis*, *E. canis*, and *Rickettsia rickettsii*", *Pathobiology* **61**, 293–296.

Duine, J.A. and Jongejan, J.A. (1989) "Quionproteins, enzymes with pyrrolo-quinoe quinone as cofactor", *Annu. Rev. Biochem.* **58**, 403–426.

Duine, J.A., Frank, J. and Jongejan, J.A. (1987) "Enzymology of quinoproteins", *Adv. Enzymol.* **59**, 69–212.

Dumler, J.S., Barbet, A.F., Bekker, C.P., Dasch, G.A., Palmer, G.H., Ray, S.C., Rikihisa, Y. and Rurangirwa, F.R. (2001) "Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and "HGE agent" as subjective synonyms of *Ehrlichia phagocytophila*", *Int. J. Syst. Evol. Microbiol.* **51**, 2145–2165.

Freiberg, C., Fellay, R., Bairoch, A., Broughton, W.J., Rosenthal, A. and Perret, X. (1997) "Molecular basis of symbiosis between *Rhizobium* and legumes", *Nature* **387**, 394–401.

Frohman, M. (1993) "Rapid amplification of complementary DNA ends for generation of full-length complementary DNAs: thermal RACE", *Methods Enzymol.* **218**, 340–356.

Goodman, J.L., Nelson, C., Vitale, B., Madigan, J.E., Dumler, J.S., Kurtti, T.J. and Munderloh, U.G. (1996) "Direct cultivation of the causative agent of human granulocytic ehrlichiosis", *N. Engl. J. Med.* **334**, 209–215.

Goodwin, P.M. and Anthony, C. (1998) "The biochemistry, physiology and genetics of PQQ and PQQ-containing enzymes", *Adv. Microb. Physiol.* **40**, 1–80.

Guo, H.C. and Roberts, J.W. (1990) "Heterogeneous initiation due to slippage at the bacteriophage 82 late gene promoter *in vitro*", *Biochemistry* **29**, 10702–10709.

Han, X. and Turnbough, Jr, C.L. (1998) "Regulation of *carAB* expression in *Escherichia coli* occurs in part through UTP-sensitive reiterative transcription", *J. Bacteriol.* **180**, 705–713.

von Heijne, G. (1986) "A new method for predicting signal sequence cleavage sites", *Nucl. Acids Res.* **14**, 4683–4690.

Jacques, J.P. and Kolakofsky, D. (1991) "Pseudo-templated transcription in prokaryotic and eukaryotic organisms", *Genes Dev.* **5**, 707–713.

Liu, C., Heath, L.S. and Turnbough, Jr., C.L. (1994) "Regulation of *pyrBI* operon expression in *Escherichia coli* by UTP-sensitive reiterative RNA synthesis during transcriptional initiation", *Genes Dev.* **8**, 2904–2912.

Machida, C., Machida, Y. and Ohtsubo, E. (1984) "Both inverted repeat sequences located at the ends of IS*1* provide promoter functions", *J. Mol. Biol.* **177**, 247–267.

Mulligan, M.E., Hawley, D.K., Entriken, R. and McClume, W.R. (1984) "Analysis of the occurrence of promoter-sites in DNA", *Nucl. Acid Res.* **12**, 789–800.

Neijssel, O.M. (1987) "PQQ-linked enzymes in enteric bacteria", *Microbiol. Sci.* **4**, 87–90.

Ohashi, N., Zhi, N., Zhang, Y. and Rikihisa, Y. (1998) "Immunodominant major outer membrane proteins of *Ehrlichia chaffeensis* are encoded by a polymorphic multigene family", *Infect. Immun.* **66**, 132–139.

Qi, F. and Turnbough, Jr., C.L. (1995) "Regulation of *codBA* operon expression in *Escherichia coli* by UTP-dependent reiterative transcription and UTP-sensitive transcriptional start site switching", *J. Mol. Biol.* **254**, 552–565.

Rawling, N.D. and Barrett, A.J. (1995) "Evolutionary families of metallopeptidases", *Methods Enzymol.* **248**, 183–228.

Ristic, M. and Holland, C.J. (1993) *Canine Ehrlichiosis* (Pergamon Press, Oxford).

Sambrock, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, New York).

Springer, A.L., Ramamoorthi, R. and Lidstrom, M.E. (1996) "Characterization and nucleotide sequence of *pqqE* and *pqqF* in *Methylobacterium extorquens* AM1", *J. Bacteriol.* **178**, 2154–2157.

Stewart, G.S., Lubinsky-Mink, S., Jackson, C.G., Cassel, A. and Kuhn, J. (1986) "pHG165: a pBR322 copy number derivative of pUC8 for cloning and expression", *Plasmid* **15**, 172–181.

Stover, C.K., Pham, X.Q., Erwin, A.L., Mizoguchi, S.D., Warrener, P., Hickey, M.J., Brinkman, F.S., Hufnagle, W.O., Kowalik, D.J., Lagrou, M., Garber, R.L., Goltry, L., Tolentino, E., Westbrock-Wadman, S., Yuan, Y., Brody, L.L., Coulter, S.N., Folger, K.R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G.K., Wu, Z. and Paulsen, I.T. (2000) "Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen", *Nature* **406**, 959–964.

Sumner, J.W., Nicholson, W.L. and Massung, R.F. (1997) "PCR amplification and comparison of nucleotide sequences from the *groESL* heat shock operon of *Ehrlichia* species", *J. Clin. Microbiol.* **35**, 2087–2092.

Tomb, J.F., White, O., Kerlavage, A.R., Clayton, R.A., Sutton, G.G., Fleischmann, R.D., Ketchum, K.A., Klenk, H.P., Gill, S., Dougherty, B.A., Nelson, K., Quackenbush, J., Zhou, L., Kirkness, E.F., Peterson, S., Loftus, B.,

Richardson, D., Dodson, R., Khalak, H.G., Glodek, A., McKenney, K., Fitzegerald, L.M., Lee, N., Adams, M.D., Venter, J.C., *et al.* (1997) "The complete genome sequence of the gastric pathogen *Helicobacter pylori*", *Nature* **388**, 539–547.

Walker, D.H. and Dumler, J.S. (1996) "Emergence of the ehrlichioses as human health problems", *Emerg. Infect. Dis.* **2**, 18–29.

Weiss, E., Dasch, G.A., Kang, Y.H. and Westfall, H.N. (1988) "Substrate utilization by *Ehrlichia sennetsu* and *Ehrlichia risticii* separated from host constituents by renografin gradient centrifugation", *J. Bacteriol.* **170**, 5012–5017.

Weiss, E., Williams, J.C., Dasch, G.A. and Kang, Y.H. (1989) "Energy metabolism of monocytic *Ehrlichia*", *Proc. Natl Acad. Sci. USA* **86**, 1674–1678.

INFECTION AND IMMUNITY, Nov. 2002, p. 5924–5930 Vol. 70, No. 11
0019-9567/02/$04.00+0 DOI: 10.1128/IAI.70.11.5924–5930.2002

Cloning and Molecular Characterization of an Immunogenic LigA Protein of *Leptospira interrogans*

Raghavan U. M. Palaniappan,[1] Yung-Fu Chang,[1]* S. S. D. Jusuf,[2] S. Artiushin,[2] John F. Timoney,[2] Sean P. McDonough,[1] Steve C. Barr,[1] Thomas J. Divers,[1] Kenneth W. Simpson,[1] Patrick L. McDonough,[1] and Hussni O. Mohammed[1]

College of Veterinary Medicine, Cornell University, Ithaca, New York 14853,[1] and Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, Kentucky 40546[2]

Received 16 January 2002/Returned for modification 7 March 2002/Accepted 24 July 2002

A clone expressing a novel immunoreactive leptospiral immunoglobulin-like protein A of 130 kDa (LigA) from *Leptospira interrogans* serovar pomona type kennewicki was isolated by screening a genomic DNA library with serum from a mare that had recently aborted due to leptospiral infection. LigA is encoded by an open reading frame of 3,675 bp, and the deduced amino acid sequence consists of a series of 90-amino-acid tandem repeats. A search of the NCBI database found that homology of the LigA repeat region was limited to an immunoglobulin-like domain of the bacterial intimin binding protein of *Escherichia coli*, the cell adhesion domain of *Clostridium acetobutylicum*, and the invasin of *Yersinia pestis*. Secondary structure prediction analysis indicates that LigA consists mostly of beta sheets with a few alpha-helical regions. No LigA was detectable by immunoblot analysis of lysates of the leptospires grown in vitro at 30°C or when cultures were shifted to 37°C. Strikingly, immunohistochemistry on kidney from leptospira-infected hamsters demonstrated LigA expression. These findings suggest that LigA is specifically induced only in vivo. Sera from horses, which aborted as a result of natural *Leptospira* infection, strongly recognize LigA. LigA is the first leptospiral protein described to have 12 tandem repeats and is also the first to be expressed only during infection. Thus, LigA may have value in serodiagnosis or as a protective immunogen in novel vaccines.

Leptospira interrogans causes leptospirosis (Weil's disease), a zoonotic disease that is prevalent in people, horses, cattle, and wild animals. The disease occurs widely in developing countries, such as Brazil and India, and is reemerging in developed countries. In addition to hepatic and renal failure, uveitis is sometimes a sequela to leptospiral infection (30). Although the incidence of human leptospirosis in the United States is relatively low, disease incidence in domestic animals has increased in recent years.

Leptospiral infection in people can range in severity from an inapparent infection to death from renal or hepatic failure (11). Infection is acquired either directly or indirectly from infected animals. In horses, the most common manifestations of infection are abortion and uveitis (29). *L. interrogans* serovar pomona type kennewicki is the predominant serovar isolated from aborted equine fetuses, whereas *L. interrogans* serovar grippotyphosa is found less frequently (7–9). The association of leptospires with equine recurrent uveitis (16) has been well documented, and the organism has been detected in ocular fluids by culture and PCR (31). In addition, Parma et al. demonstrated by Western blotting the reactivity of several bands in extracts of equine cornea and lens with antileptospiral sera (27, 28). Although there is a strong association between leptospiral infection and uveitis, the immunopathogenesis of leptospira-associated uveitis remains obscure.

Currently available leptospiral vaccines have low efficacy, are serovar specific, and generally produce only short-term immunity in domestic livestock. Efforts to identify immunogenic components of value in vaccines have resulted in characterization of 31-, 32-, 36-, and 41-kDa outer membrane proteins (12–15, 32, 33). Two of these proteins (31 and 41 kDa) function synergistically in the immunoprotection of hamsters, suggesting that an effective protein-based vaccine would contain several components (13). The search for protective immunogens is complicated by the possibility that important components may be produced only during infection. Supporting this possibility are recent studies that indicate that some immunogenic proteins of *L. interrogans* serovar pomona are upregulated at 37°C (24).

The present study was initiated to identify and characterize immunogenic *Leptospira* proteins that are expressed during infection. The gene for one such immunoreactive immunoglobulin-like 130-kDa protein (LigA) of *L. interrogans* serovar pomona type kennewicki has been characterized and shown to be expressed in vivo.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *L. interrogans* serovar pomona type kennewicki was provided by M. Donahue (Diagnostic Laboratory, Department of Veterinary Science, University of Kentucky), who isolated this strain from a case of equine recurrent uveitis. Other serovars were obtained from the American Type Culture Collection and maintained in the diagnostic laboratory at Cornell University. Leptospires were grown on PLM-5 medium (Intergen) at 30°C. Growth was monitored by dark-field microscopy. Temperature regulation was carried out as previously described (24).

* Corresponding author. Mailing address: Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853. Phone: (607) 253-3675. Fax: (607) 253-3943. E-mail: yc42@cornell.edu.

Sera. Sera were obtained from mares that had recently aborted due to leptospiral infection. These sera had high titers for *L. interrogans* serovar pomona, as determined by the microscopic agglutination test. In most cases, the diagnosis was confirmed by microscopic detection of leptospires in fetal tissues and the placenta. Rabbit antileptospiral antibody was obtained from NVSL, Iowa (1098-LEP-FAC). Antisera to LipL32 and LipL36 were kindly provided by D. A. Haake (University of California at Los Angeles).

Genomic DNA library. Genomic DNA was prepared from *L. interrogan* serovar pomona kennewicki as previously described (6). Partial restriction digestion was performed with *TSP*5091, and the digested fragments were ligated into predigested Lambda ZapII(Stratagene). Ligated DNA was packaged into Gigapack II Gold packaging extracts and stored in 0.3% chloroform. After transfection into *Escherichia coli* MRF' XL1-Blue (Stratagene), the library was plated, amplified, and screened with convalescent mare's serum according to the manufacturer's instructions.

DNA sequencing and analysis. DNA sequencing was done with an ABI model 377 automated nucleic acid sequencer at the Bioresource Center, Cornell University. Homology searches were performed with the NCBI database and BLAST (1). Secondary structure, hydrophobicity, and antigenic index predictions were obtained by using PCgene and DNAstar.

Expression of LigA in *E. coli*. *ligA* without the signal sequence (deletion of the N-terminal 31 amino acids) was amplified with primers (sense [5'-GGGTTTCATATGGCTGGCAAAAGAGGC-3'] and antisense [5'-CCCTCGAGTGGCTCCGTTTTAAT-3']) and subcloned into *Nde*I-*Xho*I sites of pET22b (Novagen, Madision, Wis.). The recombinant plasmid was transformed to *E. coli* BL21 (DE3) and expression was induced by IPTG (isopropyl-β-D-thiogalactopyranoside) as previously described (4).

A 90-kDa truncated LigA protein was subcloned into the *Xho*I-*Bam*HI sites of pET15b (Novagen) by PCR with primers (sense [5'-TCGAGGTCTCTCCAGTTTTACC-3'] and antisense [5'-GCGGATCCTGTTTTCATGTTATGGCTCC-3']). The resulting plasmid was transformed into *E. coli* BL21(DE3), and the truncated recombinant LigA fusion protein was isolated from a lysate of BL21 by affinity chromatography on His-Bind resin (Novagen).

Preparation of LigA-specific rabbit antiserum. Antiserum to a 90-kDa truncated LigA protein was prepared in adult New Zealand White rabbits. Recombinant truncate was purified from periplasmic proteins of *E. coli* Nova-Blue that contained pKS (Stratagene) encoding a 5-kb *Bam*HI-*Sal*I fragment or by affinity chromatography on Avidgel F (UniSyn Technology, Inc., Tustin, Calif.), to which immunoglobulin G (IgG) from convalescent mare's serum had been coupled. The rabbits were immunized subcutaneously with 50 μg of the 90-kDa truncated LigA mixed with complete Freund adjuvant on day 1, followed by a booster inoculum of 50 μg of protein and incomplete Freund adjuvant on days 10 and 19. On day 35, the rabbits were boosted intravenously with 50 μg of protein and then bled on day 45.

SDS-PAGE and immunoblot analysis. Purified truncated LigA protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis as previously described (3, 6). Rabbit antiserum to truncated LigA or convalescent mares' sera were used as primary antibodies. Blots were developed with peroxidase-conjugated protein G or goat anti-horse IgG conjugated to alkaline phosphatase (KPL). Reactive bands were visualized by using 4-1-chloronaphthol (0.5 mg/ml) or nitroblue tetrazolium-BCIP (5-bromo-4-chloro-3-indolylphosphate) as appropriate.

Immunohistochemistry. Immunohistochemistry was performed on normal and leptospiral infected hamster kidneys with biotin-streptavidin-horseradish peroxidase according to the manufacturer's instructions (Zymed Laboratories, South San Francisco, Calif.). The chromogen was Nova Red (Dako, Carpinteria, Calif.). The primary antibody was rabbit antiserum specific for truncated LigA and was titrated by using a twofold serial dilution from 1:10 to 1:320. Negative controls consisted of nonimmune rabbit serum diluted 1:10, 1:20, and 1:40. Anti-LipL32 was used as a positive control.

Kidneys were removed from leptospiral infected and normal hamsters euthanized as part of an unrelated research project. These tissues were immediately embedded in O.C.T. Compound (Miles, Elkhart, Ind.) and snap-frozen in 2-methyl butane (Sigma, St. Louis, Mo.) prechilled to the point of freezing in liquid nitrogen. Tissues were sectioned at 6 μm, mounted on Microscope Plus slides (Fisher Scientific), fixed in acetone for 2 min, and air dried. Endogenous peroxidase was quenched for 10 min in 0.3% hydrogen peroxide in 0.1% (wt/vol) sodium azide and rinsed for 3 min in 0.01 M phosphate-buffered saline (pH 7.6; PBS). Sections were then blocked with 10% heat inactivated goat serum for 10 min. The blocking serum was tipped off, and the primary antibody was applied for 60 min at room temperature. After three rinses in PBS, a 1:400 dilution of biotinylated goat anti-rabbit IgG was added for 20 min. Sections were rinsed three times and then incubated with a 1:400 dilution of the streptavidin-peroxidase reagent for 10 min. After the rinsing step, the chromogen-substrate mixture was added to the sections, and the reaction was monitored under the microscope until well developed or until background developed. The slides were again rinsed in PBS, counterstained lightly with Gill's #1 hematoxylin (ca. 30 s), and then rinsed in tap water. After dehydration in two changes of graded ethanol to 100% for 2 min each, the sections were cleared in four changes of 100% xylene for 2 min each and mounted with Fisher Permount.

PCR amplification of *ligA* in pathogenic serovars. Using a primer pair specific for *ligA*, PCR was performed on pathogenic serovars, including *L. interrogans* serovar pomona type kennewicki, *L. kirschneri* serovar grippotyphosa, *L. interrogans* serovar hardjo type hardjobovis, *L. interrogans* serovar icterohaemorrhagiae, and *L. interrogans* serovar canicola. The sequence of the forward primer was 5'-GGAATTCATGTTAAAGTCACTGCT-3', and that of the reverse primer was 5'-CCGCTCGAGGTTTTAATAGAGGC-3'. Amplification conditions were as previously described (5). PCR products were purified by using a gel purification kit (Qiagen) and digested with *Bam*HI and *Hin*dIII to detect restriction polymorphisms.

Enzyme-linked immunosorbent assay (ELISA). Wells of 96-well polystyrene plates (Falcon 3912 Microtest III; Becton Dickinson, Oxnard, Calif.) were coated overnight at 4°C with 0.15 μg of truncated recombinant LigA in 100 μl of PBS, washed, blocked with 2% skim milk in PBS (pH 7.2) with 0.05% Tween 20, and then incubated with a 1:100 dilution of horse serum in triplicate wells for 2 h at 37°C. After a washing step, peroxidase-conjugated protein G (1:8,000) was added (100 μl) to each well, followed by incubation for 2 h at 37°C. Finally, the plates were washed and developed with fresh substrate consisting of 0.07% *ortho*-phenylenediamine and 0.05% hydrogen peroxide in citric acid phosphate buffer (pH 5.0). After the reaction was stopped by the addition of 50 μl of 3 M sulfuric acid, the absorbance was read at 490 nm in an automated plate reader (Biotex, Winooski, Vt.).

Statistical analysis. Analysis of variance was used to determine whether there was a significant difference in the mean optical density (OD) reading for each of the sera used in the present study. Multiple comparisons by using the least-significant-difference method were performed to identify which OD mean was significantly different from the other. The analysis was performed by using Statistix software (Analytical Software, Tallahassee, Fla.).

Nucleotide sequence accession numbers. The GenBank accession number for the nucleotide sequences of *ligA* is AF368236.

RESULTS

Identification, sequencing, and expression of LigA. Screening of the *L. interrogans* genomic library with convalescent mare's serum yielded numerous positive clones, one of which contained an insert of 3,993 bp and expressed a protein that was encoded by an open reading frame of 3,675 bp (Fig. 1). The deduced sequence consisted of 1,225 amino acids with an estimated molecular mass of 129,041 Da and a pI of 6.35. An N-terminal signal sequence of 31 amino acids was predicted by using the SignalP program (25). Twelve or more tandem repeats of 90 amino acids were detected in LigA (Fig. 1B). Analysis of the sequence by using the NCBI database and BLAST revealed homology with the immunoglobulin-like domain of *E. coli* intimin (GenBank accession number AF252560), the putative invasin of *Yersinia pestis* (AJ41459), and the cell adhesion domain of *Clostridium acetobutylicum* (AE007823) (data not shown). LigA tandem repeats that showed homology with bacterial immunoglobulin-like domains (Ig11, CD:pfam02368; Ig12, CD:smart00635) are represented in Fig. 1B.

Expression of LigA in *E. coli* but not in leptospiral lysates. *E. coli* containing intact *ligA* without its signal sequence expressed LigA only after IPTG induction (Fig. 2), but LigA expression was toxic to *E. coli*, resulting in a 50-fold decrease in viability of cells (data not shown), which is similar to that found for OmpL1 of *Leptospira* (15). However, the expression of a 90-kDa truncated LigA was not toxic to *E. coli* cells (data

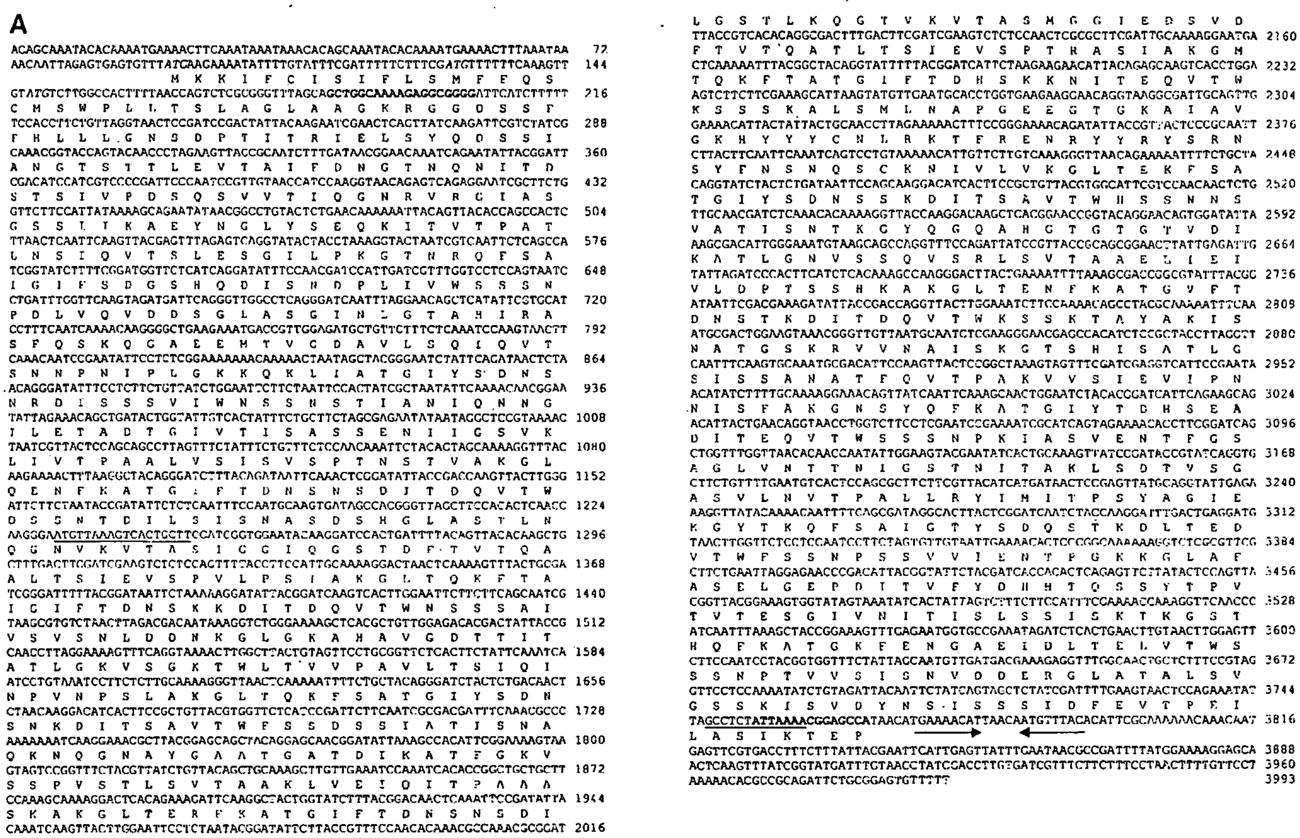

FIG. 1. (A) Nucleotide sequence of *ligA* and its deduced amino acid sequence. Italics regions are the three possible translation start codons. Bold and underlined nucleotides indicate primer annealing sites for Fig. 2 and 6, respectively. Arrows show the potential transcription termination sequence. (B) Alignment of the predicted amino acid sequences for the 12 tandem repeats and the immunoglobulin-like domain of *E. coli* intimin-binding (receptor) protein (Ig11, CD:pfam02368; Ig12, CD:smart00635). Twelve repeat sequences of a 90-amino-acid sequence include residues from 136 to 218, 224 to 310, 311 to 400, 401 to 489, 490 to 580, 581 to 670, 671 to 760, 761 to 851, 852 to 942, 943 to 1033, 1034 to 1125, and 1126 to 1216, respectively.

not shown). Immunoblotting of whole-cell lysates of *L. interrogans* serovar pomona type kennewicki grown at 30 and 37°C with LigA-specific polyclonal rabbit serum did not show any detectable level of LigA (Fig. 3). In contrast, LipL32 was expressed by cultures grown at both 30 and 37°C, whereas LipL36 was downregulated at 37°C.

LigA expression in vivo in *Leptospira*-infected hamsters. In order to examine LigA expression during leptospiral infection, immunohistochemistry was performed on kidneys from normal and leptospira-infected hamsters. LigA was expressed only in leptospira-infected hamster kidney (Fig. 4A). High-titer rabbit antileptospiral serum, as well as antiserum to LipL32, reacted with leptospires in experimentally infected kidneys (Fig. 4B and C). LipL36, which is not expressed by *L. krischneri* serovar grippotyphosa in infected hamster kidneys (2), was detected around the proximal convoluted tubules in *L. interrogans* serovar pomona-infected hamster kidney at a 1:50 dilution of antiserum to LipL36 (Fig. 4D). Preimmune rabbit serum did not react (Fig. 4E), and no immune serum reacted with normal hamster kidney (data not shown).

LigA-specific antibody in the sera of convalescent mares and aborted fetuses. All convalescent-phase sera showed strong reactivity with recombinant LigA by Western blot analysis. Negative control horse sera derived from *Borrelia burgdorferi* (5), human granuloctyic ehrlichiosis agent (HGE) infection (4), and naive horse sera were unreactive (Fig. 5). The utilization of LigA in ELISA also showed strong reactivity to the convalescent-phase sera (Table 1). The mean OD for the leptospiral positive sera (M1 to M8) was significantly different from the negative control sera (L1 to L5) and from sera obtained from HGE (E1 and E2)- and *B. burgdorferi* (N1 to N4)-infected animals ($P < 0.05$).

Detection of *ligA* in other serovars by PCR. PCR amplification revealed the presence of *ligA* in genomic DNA of the pathogenic serovars hardjo, grippotyphosa, icterohaemorrhagiae, and canicola (Fig. 6A). Restriction analysis with *Bam*HI revealed no differences in fragment patterns. However, *Hin*dIII digests revealed that *ligA* was more highly conserved in *L. interrogans* serovar pomona and *L. kirchneri* serovar grippotyphosa than in other serovars (Fig. 6B).

DISCUSSION

Characterization of bacterial antigens expressed only during infection is essential in gaining a deeper understanding of infectious diseases such as leptospirosis. Immunoscreening of

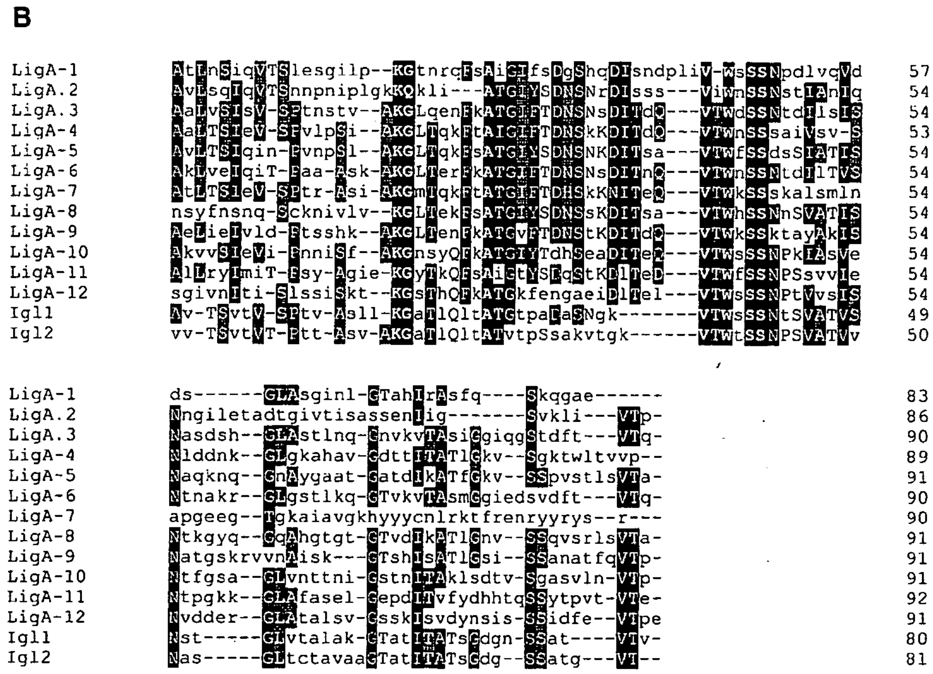

FIG. 1—*Continued.*

gene libraries with convalescent-phase serum is a powerful tool in the discovery of these in vivo-expressed immunogens, which would otherwise be difficult or impossible to identify. We have previously shown that sera from horses which aborted as a result of naturally acquired *L. interrogans* serovar Pomona type kennewicki infection recognize numerous periplasmic and outer membrane proteins, some of which are regulated by temperature (24). In the present study, an immunoscreening of a genomic library of *L. interrogans* serovar pomona type kennewicki resulted in the identification of LigA, a novel highly immunogenic protein expressed during equine infection.

LigA is mostly hydrophilic, with some hydrophobic regions located at residues 4 to 24, 306 to 326, 402 to 422, 490 to 510, and 1034 to 1054 (Fig. 1) and consists of beta sheets with a few alpha-helical regions. An Ala-Lys-Glu-Leu-Thr peptide repeat occurs at positions 416, 505, 594, and 867 corresponding to alpha-helices. LigA contains 12 or more tandem repeats of a 90-amino-acid sequence (Fig. 1B). Analysis of the nucleotide sequences by using the NCBI database and BLAST revealed no homology other than that occurring between the repeat region of LigA and the immunoglobulin-like domain of intimin-binding protein (int) of *E. coli* (17, 21, 23), the invasin of *Y. pestis* (18, 19), and a cell-binding domain of *C. acetobutylicum* (26). Based on these similarities, it is possible that LigA may also serve as an adhesin molecule. Further work is needed to clarify its role in adhesion. In the *Streptococcus* M6 protein,

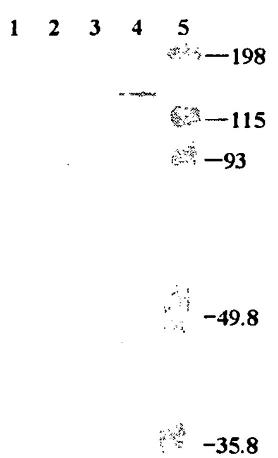

FIG. 2. Expression of LigA in *E. coli*. Whole-cell lysates of *E. coli* were subjected to SDS-PAGE, transferred to nitrocellulose, and blotted with a 1:100 dilution of rabbit antiserum to the 90-kDa truncated LigA. Lanes 1 and 2, *E. coli* with vector, pET22b only; lanes 3 and 4, *E. coli* harboring pET22b plus *ligA* construct; lanes 2 and 4, *E. coli* was induced with 0.4 mM IPTG; lane 5, prestained molecular size markers (Bio-Rad).

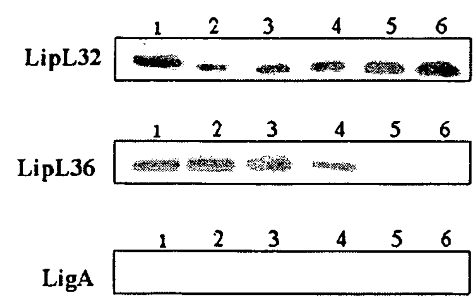

FIG. 3. LipL32 and LipL36 but not LigA expression are temperature regulated. Lane 1, whole-cell lysate of leptospires grown at 30°C; lanes 2, 3, 4, 5, and 6, cultures 2, 3, 4, 5, and 6 days old, respectively, of leptospires grown at 37°C. Each lane was loaded with ~5.0 μg of protein.

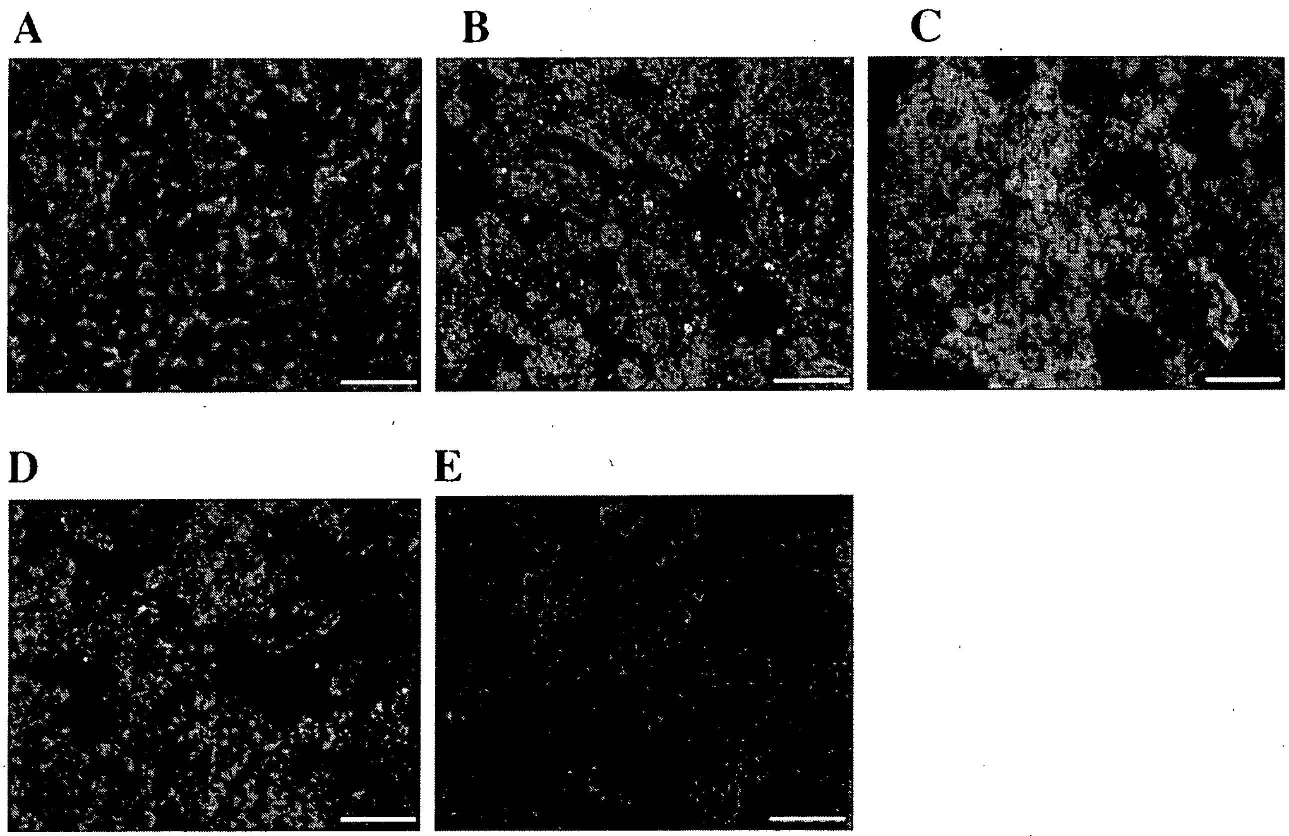

FIG. 4. LigA expression in hamsters infected with *L. interrogans* serovar pomona. Sections of kidney were treated with rabbit antiserum specific for a 90-kDa truncated LigA (A), *L. interrogans* serovar pomona (B), LipL32 (C), LipL36 (D), and with preimmune serum (E). Kidney sections from noninfected hamsters were unreactive. Bar, 67 μm.

alteration in the number of tandem repeats changes the antigenic determinants (20). In the case of parasites such as *Plasmodium*, *Leishmania*, and *Trypanosoma*, a strong antibody response is induced against immunogenic proteins containing tandem repeats, suggesting that these repetitive epitopes may camouflage vulnerable parasite antigens from a "protective" immune response (10). Overall, variation in the number of tandem repeats may cause antigenic variation, immune escape, and alteration in substrate binding properties. Thus, it is plausible that *Leptospira* evades immune response by variation in tandem repeats of LigA.

Although sera from recently aborted mares reacted strongly with the 90-kDa truncated LigA, the protein was not detectable by immunoblot in leptospira lysates cultured at 30 and 37°C. In contrast, LipL32 is expressed at both 30 and 37°C, whereas LipL36 expression is growth phase dependent (13, 24). This indicates that LigA is not expressed or thermoregulated under in vitro culture conditions.

However, immunohistochemistry with rabbit antiserum specific for a 90-kDa truncated LigA revealed expression of LigA in kidneys of infected but not uninfected hamsters. A commercially available high-titer leptopsiral antiserum showed strong reactivity to the leptospiral organisms in infected hamster kidney. Expression of LipL32 was detected both in vitro (culture) and in vivo (leptospira-infected hamster kidney), whereas LipL36 expression has been reported only in vitro (2). Our results have also confirmed the in vivo expression of LipL32. However, we noted the reactivity of LipL36 rabbit polyclonal antibody with infected hamster kidney at a 1:50 dilution. In contrast, Barnett et al. have failed to detect expression of LipL36 in *L. kirschneri* serovar grippotyphosa-infected hamster kidney. It is possible that the level of LipL36 expression differs

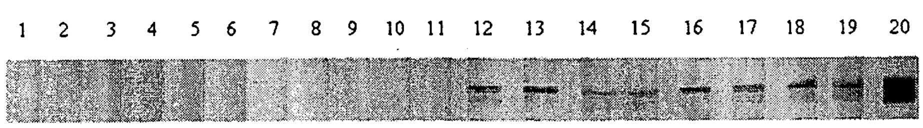

FIG. 5. Recombinant LigA protein purified by using metal affinity chromatography and subjected to SDS-PAGE separation was probed with normal horse sera (lanes 1 to 4), equine lyme disease-positive sera (lanes 5 to 9), HGE-positive sera (lanes 10 to 11), aborted mare sera (lanes 12 to 19), and rabbit serum specific for a 90-kDa truncated LigA (lane 20). Each lane was loaded with ~0.5 μg of protein.

TABLE 1. Reactivity in ELISA of rabbit antiserum to recombinant LigA, sera from horses infected with *B. burgdorferi* (L1 to L5) or *E. equi* (E1 and E2), normal horse sera (N1 to N4), and aborted mare's sera (M1 to M8) in an ELISA with a 90-kDa truncated LigA (200 ng/well)[a]

Serum	ELISA OD at serum dilution:		
	1/200	1/400	1/800
Rabbit antiserum to a 90-kDa truncated LigA	1.13	1.02	0.58
L1	0.05	0.03	0.01
L2	0.1	0.04	0.02
L3	0.03	0.02	0.02
L4	0.05	0.02	0.03
L5	0.02	0.01	0.01
E1	0.05	0.03	0.05
E2	0.08	0.05	0.04
N1	0.01	0.01	0.0
N2	0.01	0.0	0.0
N3	0.02	0.01	0.01
N4	0.03	0.03	0.01
M1	0.39	0.34	0.19
M2	0.38	0.35	0.18
M3	0.45	0.31	0.2
M4	0.6	0.56	0.27
M5	0.28	0.2	0.13
M6	0.47	0.56	0.4
M7	0.73	0.55	0.4
M8	0.56	0.5	0.42

[a] The ELISA OD values of sera from aborted mares were significantly higher ($P < 0.05$) than the values for sera from normal, *B. burgdorferi*- and *E. equi*-infected horses.

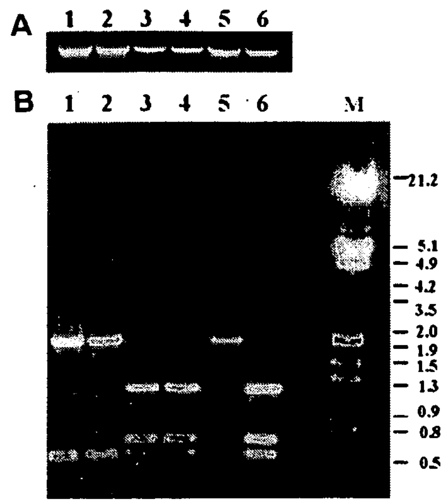

FIG. 6. Agarose gel showing PCR products and restriction analysis of *ligA* from different pathogenic serovars of *Leptospira*. (A) PCR products of *ligA*. (B) *Hin*dIII-digested PCR product of *ligA*. Lanes: 1, *L. interrogans* serovar pomona type kennewicki; 2, *L. interrogans* serovar pomona; 3, *L. interrogans* serovar hardjo; 4, *L. interrogans* serovar icterohemorrhagiae; 5, *L. kirchneri* serovar grippotyphosa; 6, *L. interrogans* serovar wolfii.

among serovars during in vivo conditions. The low level of LipL36 expression in infected hamster kidney compared to abundant expression in vitro may be a means of evading the host immune response (24). Regardless of this, these positive controls confirm that LigA is expressed only in vivo. In addition, these results suggest that LigA may be a useful antigen for differential immunodiagnosis to distinguish animals with natural infection from those that are vaccinated.

A 90-kDa protein of *Leptospira* has been previously shown to cross-react with polyclonal antiserum to an equine corneal protein (22). Immunohistochemistry, immunoprecipitation, and Western blot analysis revealed no reactivity of LigA specific antiserum with equine cornea, iris, vitreous, or lens (data not shown). Thus, LigA does not appear to share antigenic epitopes with equine ocular components and so it is clearly not the reactive protein (22).

PCR amplification of *ligA* from genomic DNA of pathogenic serovars such as hardjo, icterohaemorrhagiae, grippotyphosa, and canicola has shown that a similar sequence is widely distributed among the serovars of *L. interrogans*. However, restriction analysis with *Hin*dIII showed that the *ligA* sequence had greater similarity to that of serovars pomona and grippotyphosa than to serovars canicola and icterohaemorrhagiae. Interestingly, *L. interrogans* serovar pomona and *L. kirchneri* serovar grippotyphosa are the serovars most frequently responsible for disease in the horse.

The expression of *Leptospira* outer membrane proteins such as LipL32, LipL41, OmpL1, and LipL36 has been demonstrated in cultured organisms (12–15). Except for LipL36, these outer membrane proteins are expressed in infected hamsters. Interestingly, this is the first leptospiral protein that is not detectable in vitro (30 or 37°C) but is expressed in kidneys of infected hamsters. Importantly, identification of the in vivo-expressed LigA may lead to a better understanding of the molecular pathogenesis of leptospirosis and provide insights into the survival strategy of the organism in the host. Presumably, coordinate expression of a subset of genes during residence in vivo is necessary for survival and replication within the host. Therefore, identification of LigA that is expressed only in vivo may provide new insights for developing strategies to improve vaccination, diagnosis, and treatment protocols.

ACKNOWLEDGMENTS

This work was supported in part by the Keeneland Association, the Haggard-Davidson-McGee Equine Medicine Research Fund, the Jack Lowe Equine Research Fund, and the Harry M. Zweig Memorial Fund for Equine Research.

We thank Hsiao-Wei, Fahad Hassan, and Patricia Fisher for excellent technical assistance; David Haake for his generous gift of rabbit polyclonal antisera to LipL32 and LipL36; and Mike Donahue for *Leptospira* strains.

REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Barnett, J. K., D. Barnett, C. A. Bolin, T. A. Summers, E. A. Wagar, N. F. Cheville, R. A. Hartskeerl, and D. A. Haake.** 1999. Expression and distribution of leptospiral outer membrane components during renal infection of hamsters. Infect. Immun. **67:**853–861.
3. **Chang, Y. F., M. J. Appel, R. H. Jacobson, S. J. Shin, P. Harpending, R. Straubinger, L. A. Patrican, H. Mohammed, and B. A. Summers.** 1995. Recombinant OspA protects dogs against infection and disease caused by *Borrelia burgdorferi*. Infect. Immun. **63:**3543–3549.
4. **Chang, Y.-F., V. Novosol, E. Dubovi, S. J. Wong, F. K. Chu, C.-F. Chang, F. D. Piero, S. Shin, and D. H. Lein.** 1998. Experimental infection of the human granulocytic ehrlichiosis agent in horses. Vet. Parasitol. **78:**137–145.
5. **Chang, Y.-F., V. Novosol, S. P. McDonough, C.-F. Chang, R. H. Jacobson, T. Divers, F. W. Quimby, S. Shin, and D. H. Lein.** 2000. Experimental infection of ponies with *Borrelia burgdorferi* by exposure to *Ixodid* ticks. Vet. Pathol. **37:**68–76.

6. **Chang, Y. F., J. Shi, D. P. Ma, S. J. Shin, and D. H. Lein.** 1993. Molecular analysis of the *Actinobacillus pleuropneumoniae* RTX toxin-III gene cluster. DNA Cell Biol. **12:**351–362.
7. **Donahue, J. M., B. J. Smith, J. K. Donahoe, C. L. Rigsby, R. R. Tramontin, K. B. Poonacha, and M. A. Wilson.** 1992. Prevalence and serovars of leptospira involved in equine abortions in central Kentucky during the 1990 foaling season. J. Vet. Diagn. Investig. **4:**279–284.
8. **Donahue, J. M., B. J. Smith, K. B. Poonacha, J. K. Donahoe, and C. L. Rigsby.** 1995. Prevalence and serovars of leptospira involved in equine abortions in central Kentucky during the 1991–1993 foaling seasons. J. Vet. Diagn. Investig. **7:**87–91.
9. **Donahue, J. M., B. J. Smith, K. J. Redmon, and J. K. Donahue.** 1991. Diagnosis and prevalence of leptospira infection in aborted and stillborn horses. J. Vet. Diagn. Investig. **3:**148–151.
10. **Duncan, L. R., L. S. Gay, and J. E. Donelson.** 1991. African trypanosomes express an immunogenic protein with a repeating epitope of 24 amino acids. Mol. Biochem. Parasitol. **48:**11–16.
11. **Feigin, R. D., and D. C. Anderson.** 1975. Human leptospirosis. CRC Crit. Rev. Clin. Lab. Sci. **5:**413–467.
12. **Haake, D. A., C. I. Champion, C. Martinich, E. S. Shang, D. R. Blanco, J. N. Miller, and M. A. Lovett.** 1993. Molecular cloning and sequence analysis of the gene encoding OmpL1, a transmembrane outer membrane protein of pathogenic *Leptospira* spp. J. Bacteriol. **175:**4225–4234.
13. **Haake, D. A., G. Chao, R. L. Zuerner, J. K. Barnett, D. Barnett, M. Mazel, J. Matsunaga, P. N. Levett, and C. A. Bolin.** 2000. The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. Infect. Immun. **68:**2276–2285.
14. **Haake, D. A., C. Martinich, T. A. Summers, E. S. Shang, J. D. Pruetz, A. M. McCoy, M. K. Mazel, and C. A. Bolin.** 1998. Characterization of leptospiral outer membrane lipoprotein LipL36: downregulation associated with late-log-phase growth and mammalian infection. Infect. Immun. **66:**1579–1587.
15. **Haake, D. A., M. K. Mazel, A. M. McCoy, F. Milward, G. Chao, J. Matsunaga, and E. A. Wagar.** 1999. Leptospiral outer membrane proteins OmpL1 and LipL41 exhibit synergistic immunoprotection. Infect. Immun. **67:**6572–6582.
16. **Halliwell, R. E., T. A. Brim, M. T. Hines, D. Wolf, and F. H. White.** 1985. Studies on equine recurrent uveitis. II. The role of infection with *Leptospira interrogans* serovar pomona. Curr. Eye Res. **4:**1033–1040.
17. **Hamburger, Z. A., M. S. Brown, R. R. Isberg, and P. J. Bjorkman.** 1999. Crystal structure of invasin: a bacterial integrin-binding protein. Science **286:**291–295.
18. **Isberg, R. R., D. L. Voorhis, and S. Falkow.** 1987. Identification of invasin: a protein that allows enteric bacteria to penetrate cultured mammalian cells. Cell **50:**769–778.
19. **Jerse, A. E., and J. B. Kaper.** 1991. The *eae* gene of enteropathogenic *Escherichia coli* encodes a 94-kilodalton membrane protein, the expression of which is influenced by the EAF plasmid. Infect. Immun. **59:**4302–4309.
20. **Jones, K. F., S. K. Hollingshead, J. R. Scott, and V. A. Fishetti.** 1988. Spontaneous M6 protein size mutants of group A streptococci display variation in antigenic and opsonogenic epitopes. Proc. Natl. Acad. Sci. USA **85:**8271–8275.
21. **Kelly, G., S. Prasannan, S. Daniell, K. Fleming, G. Frankel, G. Dougan, I. Connerton, and S. Matthews.** 1999. Structure of the cell-adhesion fragment of intimin from enteropathogenic *Escherichia coli*. Nat. Struct. Biol. **6:**313–318.
22. **Lucchesi, P. M., and A. E. Parma.** 1999. A DNA fragment of *Leptospira interrogans* encodes a protein which shares epitopes with equine cornea. Vet. Immunol. Immunopathol. **71:**173–179.
23. **Luo, Y., E. A. Frey, R. A. Pfuetzner, A. L. Creagh, D. G. Knoechel, C. A. Haynes, B. B. Finlay, and N. C. Strynadka.** 2000. Crystal structure of enteropathogenic *Escherichia coli* intimin-receptor complex. Nature **405:**1073–1077.
24. **Nally, J. E., J. F. Timoney, and B. Stevenson.** 2001. Temperature-regulated protein synthesis by *Leptospira interrogans*. Infect. Immun. **69:**400–404.
25. **Nielsen, H., J. Engelbrecht, S. Brunak, and G. von Heijne.** 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. **10:**1–6.
26. **Nolling, J., G. Breton, M. V. Omelchenko, K. S. Makarova, Q. Zeng, R. Gibson, H. M. Lee, J. Dubois, D. Qiu, J. Hitti, Y. I. Wolf, R. L. Tatusov, F. Sabathe, L. Doucette-Stamm, P. Soucaille, M. J. Daly, G. N. Bennett, E. V. Koonin, and D. R. Smith.** 2001. Genome sequence and comparative analysis of the solvent-producing bacterium *Clostridium acetobutylicum*. J. Bacteriol. **183:**4823–4838.
27. **Parma, A. E., A. S. Fernandez, C. G. Santisteban, R. A. Bowden, and S. I. Cerone.** 1987. Tears and aqueous humor from horses inoculated with *Leptospira* contain antibodies which bind to cornea. Vet. Immunol. Immunopathol. **14:**181–185.
28. **Parma, A. E., C. G. Santisteban, J. S. Villalba, and R. A. Bowden.** 1985. Experimental demonstration of an antigenic relationship between *Leptospira* and equine cornea. Vet. Immunol. Immunopathol. **10:**215–224.
29. **Poonacha, K. B., J. M. Donahue, R. C. Giles, C. B. Hong, M. B. Petrites-Murphy, B. J. Smith, T. W. Swerczek, R. R. Tramontin, and P. A. Tuttle.** 1993. Leptospirosis in equine fetuses, stillborn foals, and placentas. Vet. Pathol. **30:**362–369.
30. **Rathinam, S. R., S. Rathnam, S. Selvaraj, D. Dean, R. A. Nozik, and P. Namperumalsamy.** 1997. Uveitis associated with an epidemic outbreak of leptospirosis. Am. J. Ophthalmol. **124:**71–79.
31. **Roberts, S. J.** 1958. Sequelae of leptospirosis in horses on a small farm. J. Am. Vet. Med. Assoc. **175:**803–808.
32. **Shang, E. S., M. M. Exner, T. A. Summers, C. Martinich, C. I. Champion, R. E. Hancock, and D. A. Haake.** 1995. The rare outer membrane protein, OmpL1, of pathogenic *Leptospira* species is a heat-modifiable porin. Infect. Immun. **63:**3174–3181.
33. **Shang, E. S., T. A. Summers, and D. A. Haake.** 1996. Molecular cloning and sequence analysis of the gene encoding LipL41, a surface-exposed lipoprotein of pathogenic *Leptospira* species. Infect. Immun. **64:**2322–2330.

Editor: D. L. Burns

INFECTION AND IMMUNITY, Sept. 1995, p. 3543–3549
0019-9567/95/$04.00+0

Vol. 63, No. 9

Recombinant OspA Protects Dogs against Infection and Disease Caused by *Borrelia burgdorferi*

YUNG-FU CHANG,[1]* MAX J. G. APPEL,[2] RICHARD H. JACOBSON,[1] SANG J. SHIN,[1] PETER HARPENDING,[1] REINHARD STRAUBINGER,[2] LISA A. PATRICAN,[3] HUSSNI MOHAMMED,[4] AND BRIAN A. SUMMERS[5]

Diagnostic Laboratory,[1] James A. Baker Institute for Animal Health,[2] Department of Clinical Sciences,[4] and Department of Pathology,[5] College of Veterinary Medicine, and Department of Entomology,[3] College of Agriculture and Life Science, Cornell University, Ithaca, New York 14853

Received 6 February 1995/Returned for modification 12 April 1995/Accepted 25 June 1995

Twenty-two specific-pathogen-free beagles were vaccinated with recombinant OspA (*ospA* gene derived from *Borrelia burgdorferi* B31) alone or with adjuvant (QuilA, Montanide ISA25, or aluminum hydroxide) at 6 weeks of age. Thirteen dogs were used as nonvaccinated controls with or without adjuvant. Three dogs were kept as contact controls and received neither vaccine nor challenge. Six weeks or 6 months after the first vaccination, the vaccinated (20 of 22) and nonvaccinated dogs (13) were challenged by exposure to adult ticks (*Ixodes scapularis*) naturally that were infected with *B. burgdorferi* (tick infection rate, ≥60%) and that were collected from Westchester County, N.Y. Protection from infection was evaluated by culture for *B. burgdorferi* from skin biopsies taken near the sites of tick bites. Skin biopsies were taken at monthly intervals for 3 months. *B. burgdorferi* was not isolated from any of the vaccinated dogs. In contrast, 12 of 13 control dogs challenged by exposure to the ticks were culture positive. The histopathology of the joint capsules 3 months after the challenge was used to evaluate protection from arthritis. Eight of 13 control dogs showed synovitis in single or multiple joints, while only 1 of the 22 vaccinated dogs had a single focus of mild inflammation in a single joint. At the time of the challenge, the vaccinated dogs had antibody to *B. burgdorferi*, which was demonstrable by kinetic enzyme-linked immunosorbent assay, Western blotting (immunoblotting), and a serum growth inhibition assay. The vaccinal antibody declined gradually after the challenge, especially in dogs vaccinated with OspA without adjuvants. Antibodies in the challenge control dogs were only detectable by 4 to 6 weeks after the challenge and remained at high levels until the termination of the study. Contact control dogs showed no antibody responses or histopathologic lesions and were culture negative. By Western blot analysis, antibodies to OspA first appeared in the sera of vaccinated dogs 3 weeks after the first vaccination. The absence of additional bands after the challenge suggests that infection in vaccinated dogs was blocked. Results from this study show that vaccination with recombinant OspA protected dogs against infection and disease after an experimental challenge with *B. burgdorferi* by exposure to ticks.

Lyme disease caused by *Borrelia burgdorferi* and related borrelias is one of the most common tick-transmitted diseases in the world (4, 39, 44). The spirochetes are transmitted by *Ixodes* ticks (1, 4, 9). In the United States, human Lyme disease was first discovered in Lyme, Conn. (45), and was subsequently found in dogs, horses, cattle, and cats (5, 10, 24, 29, 32, 34). The main clinical feature in humans is erythema migrans followed by joint, cardiac, or neurologic disease (44, 45). In dogs, the dominant clinical sign of Lyme borreliosis is acute lameness (24, 26, 29).

Dogs may develop arthritis after natural infection, and renal disease (21) and cardiac and neurologic disorders have been claimed (for a review, see reference 26). In a previous report, we demonstrated that experimental infections in dogs exposed to *B. burgdorferi*-infected adult ticks (*Ixodes scapularis*) resulted in an acute, recurrent lameness with fibrinopurulent arthritis (2). The dogs recovered but developed a persistent mild polyarthritis (2).

Two preparations of *B. burgdorferi* (from Fort Dodge Laboratories [8, 22, 27] and Solvay Animal Health, Inc. [unpublished data]) have been commercialized for vaccination of dogs against Lyme disease in the United States. However, Lim et al. recently reported that the vaccination of hamsters with a formalin-killed bacterin induced a destructive arthritis (28). This suggested that one or several antigens within the spirochete might play a role in the induction and activation of arthritis. Thus, it is essential to develop a vaccine which provides protection in dogs without the potential side effect of a bacterin.

Several outer surface lipoproteins from *B. burgdorferi* have been evaluated for their capacities to induce protective immunity (14, 33, 37, 38, 40, 43, 46, 48). One outer surface protein (OspA) has been reported to induce an active and protective immunity in mice (14–20, 40, 42, 43) and rhesus monkeys (36). OspA is also immunogenic in humans (23). Two brief reports described the protection of dogs of OspA (3, 11). In this paper, we report that a recombinant OspA (rOspA) is capable of inducing active immunity in specific-pathogen-free dogs which prevents infection and disease following challenge with *B. burgdorferi* delivered by infected ticks (2).

MATERIALS AND METHODS

Dogs. Thirty-eight 6-week-old specific-pathogen-free beagles from the Baker Institute colony were used. The dogs were kept in P2 isolation units. They were fed a commercial ration and water ad libitum. All dogs were observed daily for clinical signs, and their daily body temperatures were recorded. Body weights were measured weekly.

Ticks. Adult ticks (*I. scapularis*) infected with *B. burgdorferi* were collected by flagging in a forested area of Westchester County, N.Y. The ticks were maintained at the Cornell Entomology Laboratory at 94% relative humidity at 10°C

* Corresponding author. Phone: (607) 253-3900. Fax: (607) 253-3948. Electronic mail address: YC42@Cornell.edu.

TABLE 1. Isolation of *B. burgdorferi* from skin biopsies and tissues and histopathology of joints from dogs immunized with 100 μg of rOspA plus adjuvant (vaccine trial I)

Dog	Vaccine[a]	No. of days from first vaccination to challenge	Isolation of *B. burgdorferi* from: Skin biopsies by mo postchallenge 1	2	3	Tissues[c]	Histopathology of joint[b]
1	OspA + A	173	−	−	−		NL
2	OspA + A	173	−	−	−		NL
3	OspA + A	42	−	−	−		NL
4	OspA + A	42	−	−	−		NL
5	OspA + B	173	−	−	−		NL
6	OspA + B	173	−	−	−		NL
7	OspA + A + B	173	−	−	−		NL
8	OspA + A + B	173	−	−	−		NL
9	A + B	173	−	+	+	J, S	NL
10	B	173	+	+	+	S	NSM
11	A	42	−	−	−	J, M	NL
12	A	42	+	+	+	J, S	NSP
13		42	−	+	+	J, M, Me, S	NL
14		42	+	+	+	J, M, S	NSM
15		42	+	+	+	Me, S	NL
16		42	+	+	+	M, Me, S	NSM
17[d]	OspA + A		−	−	−		NL
18[d]	OspA + A		−	−	−		NL
19[e]			−	−	−		NL
20[e]			−	−	−		NL

[a] Vaccinated dogs were inoculated with 100 μg of rOpsA twice with a 3-week interval with adjuvants (A, 100 μg of QuilA; B, 100 μg of ISA25).
[b] NL, not significant lesion; NSM, nonsuppurative monoarthritis; NSP, nonsuppurative polyarthritis.
[c] Tissues positive for *B. burgdorferi*. J, joint capsules; M, muscle; Me, meninges; S, skin.
[d] Dogs 17 and 18 were vaccinated but unchallenged.
[e] Dogs 19 and 20 were neither vaccinated nor challenged (contact control).

for 2 months. To determine the percentage of ticks infected with *B. burgdorferi*, 20 male or female ticks were ground and cultured individually in BSK-II medium with 8 μg of kanamycin per ml and 50 μg of rifampin per ml as previously described (2, 7, 41). The cultures were then examined over a 6-week period for *B. burgdorferi* by dark-field microscopy and by indirect immunofluorescence. They showed a 60 to 80% infectivity rate.

Overexpression of rOspA. rOspA derived from *B. burgdorferi* B31 was overexpressed by a T7 promoter and purified by immobilized metal ion affinity chromatography as previously described (7). Purified rOspA was kept at −20°C until used. rOspA supplied by Connaught Laboratories Inc., Swiftwater, Pa., for vaccine trial II was prepared as described previously (13).

Vaccination of dogs. We evaluated the immunogenicity and efficiency of the recombinant vaccine in three trials.

(i) Vaccine trial I. Twenty 6-week-old male and female beagles were used in this study. The dogs were randomly allotted to a vaccination group (10 dogs) and a nonvaccinated control group (10 dogs). Each dog in the vaccinated group was injected intramuscularly twice with a 3-week interval with 100 μg of rOspA in adjuvant. Six dogs received 100 μg of QuilA in a 1-ml emulsion as an adjuvant (Accurate Chemistry and Scientific Company, Westbury, N.Y.), two dogs received 25 μg of Montanide ISA25 (Seppic, Paris, France), and two dogs received both 100 μg of QuilA and 25 μg of Montanide ISA25. Control dogs were injected intramuscularly twice with the adjuvants only, two dogs were injected with 100 μg of QuilA, one dog received 25 μg of Montanide ISA25, and one dog received a combination of both adjuvants (100 μg of QuilA plus 25 μg of Montanide ISA25). Four additional nonvaccinated dogs (Table 1) were challenged by exposure to ticks; two dogs without any injection or challenge were maintained as contact control dogs but were kept in the same unit with the infected dogs. Six vaccinated dogs and two control dogs were challenged 6 months after the first vaccination. Two vaccinated dogs and the contact control dogs were not challenged. All other dogs were challenged 6 to 7 weeks after the first vaccination (Table 1).

(ii) Vaccine trial II. In order to perform a titration of the protective antigen dose and to compare the capacities of the adjuvants to elicit maximum protection for humans, six 6-week-old beagles were allotted to a vaccination group (four dogs) and a nonvaccination control group (two dogs). Each dog in the vaccine group was given two subcutaneous injections of 10 μg of rOspA in 10 μg of 1% aluminum hydroxide (rOspA and the adjuvant were supplied by Connaught

TABLE 2. Isolation of *B. burgdorferi* from skin biopsies and tissues and histopathology of joints from dogs immunized with 10 μg of rOspA plus adjuvant (vaccine trial II)

Dog	Vaccine[a]	No. of days from first vaccination to challenge	Isolation of *B. burgdorferi* from: Skin biopsies by mo postchallenge 1	2	3	Tissues[c]	Histopathology of joint[b]
21	OpsA + H	42	−	−	−		NL
22	OpsA + H	42	−	−	−		NL
23	OpsA + H	42	−	−	−		NL
24	OpsA + H	42	−	−	−		NSM
25		42	+	+	+	J, M, P, S	NSM
26		42	+	+	+	J, M, P, S	NSP

[a] Vaccinated dogs were inoculated with 10 μg of rOspA plus aluminum hydroxide (H) twice with a 3-week interval.
[b] NL, not significant lesion; NSM, nonsuppurative monoarthritis; NSP, nonsuppurative polyarthritis.
[c] Tissues positive for *B. burgdorferi*. J, joint; M, muscle; P, pericardium; S, skin.

TABLE 3. Isolation of *B. burgdorferi* from skin biopsies and tissues and histopathology of joints from dogs immunized with 100 μg of rOspA without adjuvant (vaccine trial III)

Dog	Vaccine[a]	No. of days from first vaccination to challenge	Isolation of *B. burgdorferi* from: Skin biopsies by mo postchallenge 1	2	3	Tissues[c]	Histopathology of joint[b]
27	OspA	49	–	–	–		NL
28	OspA	49	–	–	–		NL
29	OspA	49	–	–	–		NL
30	OspA	49	–	–	–		NL
31	OspA	49	–	–	–		NL
32	OspA	49	–	–	–		NL
33	OspA	49	–	–	–		NL
34	OspA	49	–	–	–		NL
35		49	+	+	+	M, S	NL
36		49	+	+	+	J, M, S	NSM
37		49	+	+	+	J, M, S	NSM
38[d]			–	–	–		NL

[a] Vaccinated dogs were inoculated twice with 100 μg of rOspA with a 3-week interval.
[b] NL, not significant lesion; NSM, nonsuppurative monoarthritis.
[c] Tissues positive for *B. burgdorferi*. J, joints; M, muscle; S, skin.
[d] Dog 38 was neither vaccinated nor challenged (contact control).

Laboratories) with a 3-week interval. The two control dogs were not inoculated. All dogs were challenged by exposure to ticks (Table 2).

(iii) Vaccine trial III. Twelve 6-week-old beagles were used. Each of eight dogs in the vaccine group was injected intramuscularly twice with a 3-week interval with 1 ml of 100 μg of rOspA without adjuvant. Four dogs were kept as nonvaccinated controls. All eight dogs in the vaccinated group and three of four dogs in the nonvaccinated group were challenged by exposure to ticks. One nonvaccination dog was kept as a contact control (Table 3).

Challenge infection of dogs after vaccination. The challenge infection of the dogs was made by placing 15 female and 7 male adult ticks (*I. scapularis*) onto the clipped side of a dog as previously reported (2). The infectivity rate of the ticks was ≥60%. The ticks were allowed to feed and engorge for 6 days, when at least 50% of the female ticks were fully engorged.

Collection of serum and tissue samples. Serum samples were obtained from each dog at the time of the first and second vaccinations, at the challenge, and at 2-week intervals for 3 months thereafter. The sera were tested by Western blotting (immunoblotting), kinetic enzyme-linked immunosorbent assays (KELA), and *B. burgdorferi* growth inhibition assays. After the dogs were exposed to the ticks, they had skin biopsies taken at monthly intervals for the isolation of spirochetes. Three months after the challenge, all dogs were euthanized and tissues were removed for the culture of *B. burgdorferi* and for histopathology.

Isolation of *B. burgdorferi*. To test for protection from infection, attempts to isolate *B. burgdorferi* from skin biopsies and from various tissues were made. Samples from skin punch biopsies (diameter, 4 mm) collected at monthly intervals after exposure to the ticks and pieces of tissue (approximately 0.2 to 1 gm) from necropsy were homogenized in 3 ml of BSK-II medium in a tissue homogenizer (stomacher; Tekmar, Cincinnati, Ohio) and then transferred to 27 ml of prewarmed BSK-II medium. At necropsy, the following tissues were removed aseptically for culture: four limb muscles, six joint capsules (right and left stifle, elbow, and shoulder joints), the pericardium, the peritoneum, lymph nodes, and meninges (dura mater). The cultures were checked weekly for up to 6 weeks for the presence of *B. burgdorferi* by dark-field examination and by the indirect fluorescence assay.

Serology: KELA, immunoblots, and growth inhibition tests. The KELA for measuring the levels of serum antibody to *B. burgdorferi* was described previously (2, 41). Each unit of slope was designated as a KELA unit. Western blot confirmation of sera indicated that the cutoff separating negative from positive sera was 100 KELA units (2, 41). Briefly, diluted serum was added to duplicate wells in microtiter plates containing antigens of French-pressed *B. burgdorferi* lysate. Bound antibody was detected by using second antibodies of a goat anti-canine antibody of heavy- and light-chain specificity conjugated to horseradish peroxidase (Cappel Research Products, Durham, N.C.). Color development with the chromogen tetramethylbenzidine with H_2O_2 as a substrate was measured kinetically and expressed as the slope of the reaction rate between the enzyme and the substrate solution.

The procedure for the Western blot analysis was previously described (2, 7). French-pressed *B. burgdorferi* lysate was used as an antigen and was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7). Western blot analysis was performed in a miniblotter (2). Test sera from experimental animals were used as a first antibody, and goat anti-dog immunoglobulin G conjugated to horseradish peroxidase was used as a second antibody.

The growth inhibition assay was done as described elsewhere (6, 39, 47). Briefly, serial dilutions of serum in microtitration plates were incubated with 10^6 live *B. burgdorferi* per ml in BSK-II medium for 30 min, and guinea pig complement was then added. The microtitration plates were sealed and incubated at 34°C for 4 to 6 days. Bacterial growth was measured as a function of the pH, which was indicated by a color change from red to yellow and which was determined with a microplate reader set at 570 nm.

Gross pathology and histopathology. All vaccinated and nonvaccinated dogs were euthanized approximately 3 months after exposure to the ticks and were examined for gross and histopathologic lesions to document protection from disease. The following tissues were fixed in 10% neutral formalin: joint capsules (elbow, shoulder, stifle, and tarsus) and lymph nodes. Tissues were embedded in paraffin wax, sectioned, and stained by conventional methods for histopathologic evaluation.

Statistical analysis. The protective effect of the vaccine against the development of infection and disease while controlling for different times of challenge was evaluated by logistic regression analysis with BMDP statistical software (BMDP Statistical Software Inc., Los Angeles, Calif.). The analysis was performed for each adjuvant separately. To evaluate the effect of using the aluminum hydroxide adjuvant, Fisher's exact test was used.

RESULTS

Clinical signs. None of the vaccinated dogs showed any clinical signs (lameness, anorexia, or depression) or elevated body temperature following vaccination or challenge by exposure to the ticks. Local reactions to adjuvant inoculation were mild swelling and reddening, which disappeared by 3 days after inoculation. Among the nonvaccinated control dogs, only one dog (dog 36) became lame for a 4-day period 3 months after the challenge. Thirteen nonvaccinated control dogs had sporadic elevated body temperatures for 1 or 2 days. In contrast to the vaccinated dogs, nonvaccinated control dogs were frequently reluctant to get up and appeared depressed. Anorexia and loss in body weight were not seen in any of the dogs. Clinical signs and temperature elevation were not found in the three contact control dogs and in the two vaccinated dogs that were not challenged.

Isolation of *B. burgdorferi*. *B. burgdorferi* was not isolated from any of the skin biopsies taken from the vaccinated dogs at monthly intervals after the challenge or from the skeletal muscles, joint capsules, pericardium and peritonium samples, lymph nodes, and meninges collected at necropsy. Twelve of 13 dogs in the nonvaccinated, challenged control group were culture positive (Tables 1 to 3). The skin and tissue samples from the two dogs vaccinated with 100 μg of rOspA plus 100 μg of

QuilA that were/not challenged and from nonvaccinated contact control dogs were all culture negative (Table 1 and 3). There was a significant difference between the vaccinated and nonvaccinated groups with regard to the development of challenge infections ($P < 0.05$). There was no significant difference among dogs of the vaccinated group with respect to the intervals between vaccination and challenge or to the different adjuvants ($P > 0.05$).

Serology. At the time of the challenge, all vaccinated dogs had antibody to *B. burgdorferi* as determined by KELA, with antibody levels of between 300 to 600 KELA units which gradually declined after the challenge (Fig. 1). Antibody levels in dogs vaccinated with rOspA without adjuvant declined more rapidly compared with those in dogs vaccinated with rOspA with adjuvants (Fig. 1). Antibodies (as determined by KELA) in the nonvaccinated, challenged control dogs were detectable by 4 to 6 weeks after challenge (Fig. 1). Western blot analysis showed OspA antibody in the 32-kDa region 3 weeks after the first vaccination. Bands became denser after the second vaccination. Bands also appeared in the 20-kDa and 62-kDa regions. Additional bands were not seen after the challenge with the ticks. In contrast, multiple bands were seen for the nonvaccinated control dogs by 4 to 6 weeks after exposure to the infected ticks (Fig. 2). The *B. burgdorferi* growth inhibition assay with sera from the vaccinated dogs showed titers of 1:40 to 1:2,560 at the time of the challenge, with no statistically significant difference among the formulations of the vaccine with different adjuvants ($P > 0.05$). However, like the levels of antibodies determined by KELA, those determined by the growth inhibition assay declined more rapidly in dogs that were vaccinated with rOspA without adjuvant (data not shown). Sixty days after the second vaccination, antibody titers by the growth inhibition assay had declined to levels of $\leq$1:40 in dogs that had received 100 μg of rOspA without adjuvant. In contrast, dogs that received 100 μg of rOspA together with QuilA or ISA25 had antibody titers of $\geq$1:160 by 6 months after the first vaccination. Three of 4 dogs vaccinated with 10 μg of rOspA plus aluminum hydroxide had antibody titers of $\geq$1:160 by 4 months after the second vaccination. Sera from the control dogs did not show growth inhibition before the challenge (Fig. 3). The contact control dogs did not develop titers by either KELA or the growth inhibition assay (data not shown).

Histopathology. Histopathologic examinations were performed on joint tissues from the elbow, shoulder, stifle, and tarsal joints (eight specimens per dog). Of 13 nonvaccinated control dogs challenged by exposure to ticks, 8 showed a nonsuppurative synovitis characterized by a perivascular-to-diffuse infiltrate with plasma cells and lymphocytes (Fig. 4). This infiltrate was commonly seen close to the synovial surface but sometimes was deeper in the joint capsule. In affected joints, multiple foci of these mononuclear cells were seen; a few dogs showed inflammation in more than one joint (polyarthritis). With the exception of one focal joint lesion in one dog (dog 24) (Table 2), no histopathologic joint lesions were found in the vaccinated dogs. The two dogs that were given only rOspA (dogs 17 and 18) (Table 1) but that were not challenged showed no joint lesions. Lymph nodes, especially the axillary or prescapular, were moderately to markedly increased in size on the side of the dog where the challenge with the ticks had occurred. There was mild follicular and parafollicular hyperplasia in the vaccinated dogs. The two vaccinated but unchallenged dogs (dogs 17 and 18) also showed a mild follicular and parafollicular hyperplasia. The follicular and parafollicular hyperplasia was more pronounced in nonvaccinated dogs. Large follicles were often formed within both the cortex and medulla of the lymph node. In this study, all combinations of rOspA (10

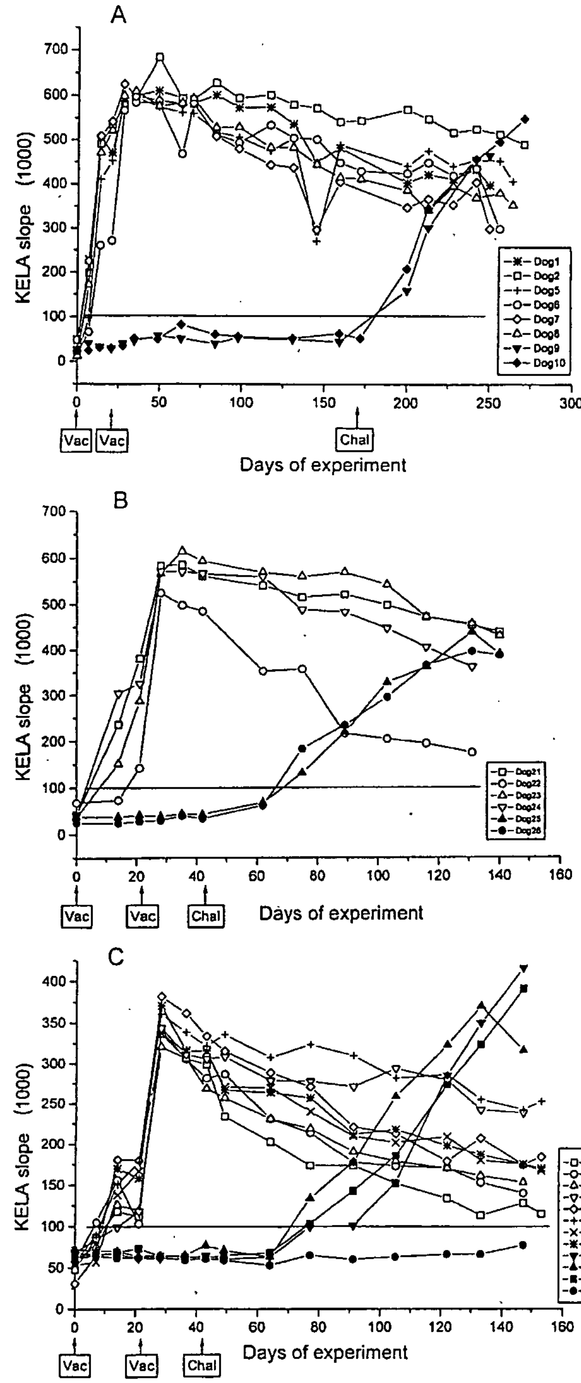

FIG. 1. Antibody levels in vaccinated and unvaccinated dogs as determined by KELA. The line at 100 KELA units represents the cutoff between positive and negative sera. Open symbols indicate vaccinated dogs, and solid symbols indicate nonvaccinated dogs. (A) Vaccinated dogs were inoculated twice with 100 μg of rOspA plus adjuvants intramuscularly with a 3-week interval and were challenged with *B. burgdorferi*-infected ticks 6 months later. (B) Vaccinated dogs were inoculated twice with 10 μg of rOspA plus adjuvant subcutaneously with a 3-week interval and were challenged with *B. burgdorferi*-infected ticks at 42 days after the first vaccination. (C) Vaccinated dogs were inoculated twice with 100 μg of rOspA without adjuvants intramuscularly with a 3-week interval and were challenged with *B. burgdorferi*-infected ticks at 49 days after the first vaccination. All unvaccinated dogs in each group were kept with vaccinated dogs and were challenged at the same time. Vaccination (Vac) and challenge (Chal) times are indicated by arrows.

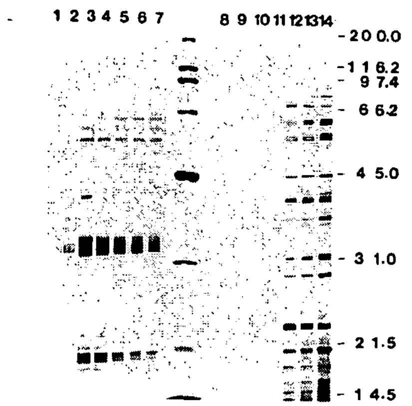

FIG. 2. Western blot analysis of the antibody response of a dog (dog 4) vaccinated with 100 μg of rOspA and QuilA (lanes 1 to 7; see also Table 1) and of an unvaccinated dog (dog 14) (lanes 8 to 14; see also Table 1). Lane 1, preimmune serum; lane 2, 3 weeks after the first vaccination; lane 3, 6 weeks after the first vaccination (at the time of the challenge with the ticks); lane 4, 10 weeks after the first vaccination; lane 5, 13 weeks after the first vaccination; lane 6, 16 weeks after the first vaccination; lane 7, 20 weeks after the first vaccination. Lanes 8 to 14 correspond to lanes 1 to 7 but are for unvaccinated dog 14. The molecular weight marker lane is at the center of the figure, between lanes 7 and 8. The biotinylated SDS-PAGE standard broad-range molecular markers (Bio-Rad Laboratories, Richmond, Calif.) were used. The numbers at the right indicate molecular weights.

or 100 μg) plus various adjuvants (QuilA, ISA25, and aluminum hydroxide) provided protection from infection and disease.

DISCUSSION

Our criterion for the protection of dogs from infection was the failure to isolate live *B. burgdorferi* from monthly skin biopsies after the challenge with ticks and from a variety of tissues taken by necropsy at 3 months after the exposure. We did not use PCR to detect *B. burgdorferi* DNA, because positive results would not differentiate between viable and nonviable organisms. Except for one dog in trial I (dog 11) (Table 1), *B. burgdorferi* was isolated from skin biopsies or other tissues from all nonvaccinated control dogs challenged by the exposure to ticks. In contrast, attempts to isolate the organism were negative for all vaccinated dogs (Tables 1 to 3).

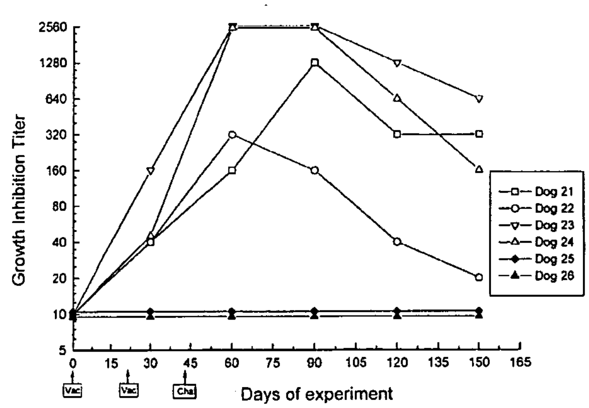

FIG. 3. Antibody titers from the vaccinated and unvaccinated dogs of vaccine trial II as determined by the growth inhibition assay. Open symbols indicate vaccinated dogs, and solid symbols indicate nonvaccinated dogs. Sera were from the same group of dogs as that reported on in Fig. 1B. Vaccination (Vac) and challenge (Chal) times are indicated by arrows.

In our earlier studies of canine Lyme disease (2), we found that clinical signs of lameness with joint swelling and stiffness were seen when puppies at 6 weeks of age were exposed to infected ticks. We have seen lameness or other clinical signs in only about 5% of control dogs challenged when 12 weeks of age or older (2). That percentage corresponds with the incidence of Lyme disease in dogs in the field (25). Accordingly, one of our criteria for protection from the disease was the presence or absence of histopathologic changes in joint tissues at 3 months after the challenge. Mono- or polyarthritis was found in 8 of 13 nonvaccinated control dogs exposed to infected ticks. In contrast, except for one lesion in one joint of one dog (dog 24) (Table 2), vaccinated dogs were free of joint lesions. Whether the single lesion was caused by the vaccination, the challenge, or other undefined causes remains unknown. Nevertheless, the possibility of OspA-induced joint lesions cannot be ruled out. It seems unlikely that this single focal lesion was caused by *B. burgdorferi*, as all tissues cultured from vaccinated dogs were negative. The lymph nodes from vaccinated, unchallenged dogs (dogs 17 and 18) (Table 1) also showed a mild follicular and parafollicular hyperplasia. Whether the mild-to-moderate hyperplasia observed in vaccinated dogs reflected a response to the rOspA, the adjuvant, or both elements is unknown. We speculated that the adjuvant is the more likely inciting factor.

High antibody levels in vaccinated dogs prior to the challenge, as determined by KELA, apparently correlated with protection against the challenge (Fig. 1). However, the antibodies in vaccinated dogs reflected antibody to the 32-kDa OspA, which is not present in the dogs after the exposure to the ticks (2). Western blots had reliable markers associated with protection. In all vaccinated dogs, Western blots expressed a wide band in the 32-kDa OspA region. Additionally, we saw weaker bands in the 20-kDa region that might indicate breakdown products of OspA. We also saw bands in the 60-kDa region which may reflect antibody responses to a dimer or polymer of OspA. The Western blot pattern for vaccinated dogs did not change after the challenge, suggesting that challenge infections did not become established in these vaccinated dogs. The 32-kDa bands waned with time (Fig. 2, lanes 4 to 7), suggesting that a drop in vaccinal antibody levels occurred as a function of time.

A correlation between serum growth inhibition of *B. burgdorferi* and protection from infection was found. By the growth inhibition assay, all the vaccinated dogs had high antibody titers after vaccination (Fig. 3) that gradually declined after the challenge with the ticks. It is possible that antibodies neutralized *B. burgdorferi* in the tick gut and prevented migration to the salivary gland and into the host as reported previously (17). We have not attempted to isolate *B. burgdorferi* from ticks after their engorgement on vaccinated dogs. Following the challenge, the nonvaccinated dogs lacked antibodies to *B. burgdorferi* as determined by the growth inhibition assay, even though they showed high antibody titers by KELA (Fig. 1). Western blot analysis also showed a strong antibody response to the *B. burgdorferi* antigens (Fig. 2, lanes 12 and 13). However, no significant anti-OspA antibodies can be detected with these sera (47). This indicated that the anti-OspA antibodies are a critical factor in the inhibition of *B. burgdorferi* growth.

The use of adjuvant is important in combination with rOspA. Although dogs vaccinated with rOspA without adjuvant were protected when challenged 4 weeks after the last vaccination (dogs 27 to 34) (Table 3), antibodies (by both assays) declined rapidly within 2 months of the challenge. In contrast, dogs

FIG. 4. Histologic appearance of synovial membranes from vaccinated and nonvaccinated dogs exposed to *B. burgdorferi*-infected ticks. (A) Dog 21. In this vaccinated dog, the synovial membrane is normal. The surface is slightly undulating with occasional folds. Hematoxylin-eosin stain was used. Magnification, ×250. (B) Dog 16. In this nonvaccinated control dog, there is synovial inflammation. The synoviocytes have proliferated into papillae (arrows), and a mononuclear cell infiltration is seen below the synovial surface. Hematoxylin-eosin stain was used. Magnification, ×250.

vaccinated with rOspA plus QuilA and/or ISA25 were protected from infection when they were challenged 6 months after the vaccination. Antibodies in these dogs remained high by both assays. Our data confirm the results reported by Ma et al. (31).

The heterogeneity of the OspA proteins in different *Borrelia* species has been reported mainly in Europe and Asia (4, 12, 35, 49). This aspect has to be considered with regard to immunization against Lyme disease. With few exceptions, one serotype seems to prevail in the United States (4, 12, 35). We, therefore, have not addressed the question of heterogeneity in our vaccine trial. However, as more data on *B. burgdorferi* serotype diversity in the United States are accumulating, this problem may have to be addressed (30).

The OspA subunit vaccine is a good candidate vaccine for dogs and is currently being tested for safety and immunogenicity in humans (23). The dog has proved to be a good model for human Lyme arthritis (2). A vaccine study in dogs, therefore, would also be valuable in the evaluation of human Lyme vaccines. Active and passive protection of mice by OspA against infection with *B. burgdorferi* when challenged by needle inoculation or exposure to ticks has been reported (14–20, 38, 40, 43). Our data show that dogs can also be protected by rOspA vaccination.

In summary, a rOspA subunit vaccine protected dogs against *B. burgdorferi* infection and disease. Further studies of the duration of protection after vaccination, safety, and cross protection against the possible heterogeneous OspA structures that may be found among new *B. burgdorferi* strains isolated in the United States are needed (30).

ACKNOWLEDGMENTS

We are grateful to Helen Bell for administrative assistance and to Patti Easton and Mary Beth Matychak for technical assistance. Lennart Krook kindly assisted in the preparation of the illustrations of the joint tissue.

This work was supported by grants from the Morris Animal Foundation, the Connaught Laboratories, and the Cornell alumni fund.

REFERENCES

1. **Anderson, J. F.** 1989. Epizootiology of *Borrelia* in *Ixodes* tick vectors and reservoir hosts. Rev. Infect. Dis. **11:**S1415–1459.
2. **Appel, M. J., S. Allan, R. H. Jacobson, T. L. Lauderdale, Y. F. Chang, S. J. Shin, and B. A. Summers.** 1993. Experimental Lyme borreliosis in dogs produces arthritis and persistent infection. J. Infect. Dis. **167:**651–664.
3. **Appel, M. J. G., Y. F. Chang, R. H. Jacobson, S. J. Shin, L. A. Patrican, B. A. Summers, and R. K. Straubinger.** 1994. Protection of SPF dogs from experimental infection and disease with *Borrelia burgdorferi* by immunization with OspA, p. 119–122. *In* R. Cevenini, V. Sambri, and M. La Placa (ed.), Advance in Lyme borreliosis research. Proceedings of the VIth International Conference on Lyme Borreliosis. 19 to 22 June 1994, Bologna, Italy.
4. **Baranton, G., D. Postic, I. Saint Girons, P. Boerlin, J.-C. Piffaretti, M. Assous, and P. A. D. Grimont.** 1992. Delineation of *Borrelia burgdorferi* sensu stricto, *Borrelia garnii* sp. nov., and group VS461 associated with Lyme borreliosis. Int. J. Syst. Bacteriol. **42:**378–383.
5. **Burgess, E. C.** 1988. *Borrelia burgdorferi* infection in Wisconsin horses and cows, p. 235–243. *In* J. L. Benach (ed.), Lyme disease and related disorders. The New York Academy of Sciences, New York.
6. **Callister, S. M., R. F. Schell, K. L. Case, S. D. Lovrich, and S. P. Day.** 1993. Characterization of the borreliacidal antibody response to *Borrelia burgdor-*

feri in humans: a serodiagnostic test. J. Infect. Dis. 167:158–164.

7. **Chang, Y. F., T. L. Lauderdale, W. Y. Lee, S. J. Shin, R. H. Jacobson, M. J. Appel, and D. H. Lein.** 1992. Expression and secretion of outer surface protein (OspA) of *Borrelia burgdorferi* from *E. coli*. FEMS Microbiol. Lett. 109:297–302.
8. **Chu, H.-J., L. G. Chavez, B. M. Blumer, R. W. Sebrring, T. L. Wasmoen, and W. M. Acree.** 1992. Immunogenicity and efficacy study of a commercial *Borrelia burgdorferi* bacterin. J. Am. Vet. Med. Assoc. 201:403–411.
9. **Ciesielski, C. A., L. E. Markowitz, R. Horsley, A. W. Hightower, H. Russell, and C. V. Broome.** 1988. The geographic distribution of Lyme disease in the United States. p. 283–288. *In* J. L. Benach (ed.), Lyme disease and related disorders. The New York Academy of Sciences, New York.
10. **Cohen, N. D., and D. Cohen.** 1990. Borreliosis in horses: a comparative review. Compend. Contin. Educat. Practic. Vet. 12:1449–1458.
11. **Coughlin, R. T., D. Fish, T. Marther, J. Ma, C. Pavia, and P. Bulger.** 1994. Protection of dogs from Lyme disease with a vaccine containing OspA, OspB, and the saponin adjuvant, p. 115–118. *In* R. Cevenini, V. Sambri, and M. La Placa (ed.), Advance in Lyme borreliosis research. Proceedings of the VIth International Conference on Lyme Borreliosis. 19 to 22 June 1994, Bologna, Italy.
12. **Dykhuizen, D. E., D. S. Polin, J. J. Dunn, B. Wilske, V. Preac-Mursic, R. J. Dattwyler, and B. J. Luft.** 1993. *Borrelia burgdorferi* is clonal: implications for taxonomy and vaccine development. Proc. Natl. Acad. Sci. USA 90:10163–10167.
13. **Erdile, L. F., M.-A. Brandt, D. J. Warakomski, G. J. Westrack, A. Sadziene, A. G. Barbour, and J. P. Mays.** 1993. Role of attached lipid in immunogenicity of *Borrelia burgdorferi* OspA. Infect. Immun. 61:81–90.
14. **Fikrig, E., S. W. Barthold, F. S. Kantor, and R. A. Flavell.** 1990. Protection of mice against the Lyme disease agent by immunizing with recombinant OspA. Science 250:553–556.
15. **Fikrig, E., S. W. Barthold, F. S. Kantor, and R. A. Flavell.** 1992. Long-term protection of mice from Lyme disease by vaccination with OspA. Infect. Immun. 60:773–777.
16. **Fikrig, E., S. W. Barthold, N. Marcantonio, K. Deponte, F. S. Kantor, and R. A. Flavell.** 1992. Roles of OspA, OspB, and flagellin in protective immunity to Lyme borreliosis in laboratory mice. Infect. Immun. 60:657–661.
17. **Fikrig, E., S. R. Telford, S. W. Barthold, F. S. Kantor, A. Spielman, and R. A. Flavell.** 1992. Elimination of *Borrelia burgdorferi* from vector ticks feeding on OspA-immunized mice. Proc. Natl. Acad. Sci. USA 89:5418–5421.
18. **Fikrig, J., S. W. Barthold, and R. A. Flavell.** 1993. OspA vaccination of mice with established *Borrelia burgdorferi* infection alters disease but not infection. Infect. Immun. 61:2552–2557.
19. **Fikrig, J., S. W. Barthold, F. S. Kantor, and R. A. Flavell.** 1993. Protection of mice from Lyme borreliosis by oral vaccination with *Escherichia coli* expressing OspA. J. Infect. Dis. 164:1224–1227.
20. **Gern, L., O. Rais, C. Capiau, P. Hauser, Y. Lobet, E. Simoen, P. Voet, and J. Petre.** 1994. Immunization of mice by recombinant OspA preparations and protection against *Borrelia burgdorferi* infection induced by *Ixodes ricinus* tick bite. Immunol. Lett. 39:249–258.
21. **Grauer, G. F., E. C. Burgess, A. J. Cooley, and J. H. Hagee.** 1988. Renal lesions associated with *Borrelia burgdorferi* infection in a dog. J. Am. Vet. Med. Assoc. 193:237–239.
22. **Kazmierczak, K. J., and F. E. Sorhage.** 1993. Current understanding of *Borrelia burgdorferi* infection, with emphasis on its prevention in dogs. J. Am. Vet. Med. Assoc. 203:1524–1528.
23. **Keller, D., F. T. Koster, D. H. Marks, P. Hosbach, L. F. Erdile, and J. P. Mays.** 1994. Safety and immunogenicity of a recombinant outer surface protein A Lyme vaccine. JAMA 271:1764–1768.
24. **Kornblatt, A. N., P. H. Urband, and A. C. Steere.** 1985. Arthritis caused by *Borrelia burgdorferi* in dogs. J. Am. Vet. Med. Assoc. 186:960–964.
25. **Levy, S., and L. A. Magnarelli.** 1992. Relationship between development of antibodies to *Borrelia burgdorferi* in dogs and the subsequent development of limb-joint borreliosis. J. Am. Vet. Med. Assoc. 200:344–347.
26. **Levy, S. A., S. W. Barthold, D. M. Daubach, and T. L. Wasmoen.** 1993. Canine Lyme borreliosis. Compend. Contin. Educat. Practic. Vet. 15:833–848.
27. **Levy, S. A., B. Lissman, and C. Ficke.** 1993. Performance of a *Borrelia burgdorferi* bacterin in borreliosis-endemic areas. J. Am. Vet. Med. Assoc. 202:1834–1838.
28. **Lim, L. C. L., D. M. England, B. K. DuChateau, N. J. Glowacki, J. R. Creson, S. D. Lovrich, S. M. Callister, D. A. Jobe, and R. F. Schell.** 1994. Development of destructive arthritis in vaccinated hamsters challenged with *Borrelia burgdorferi*. Infect. Immun. 62:2825–2833.
29. **Lissman, B. A., E. M. Bosler, H. Camay, B. G. Ormiston, and J. L. Benach.** 1984. Spirochete-associated arthritis (Lyme disease) in a dog. J. Am. Vet. Med. Assoc. 185:219–220.
30. **Lovrich, S. D., S. M. Callister, L. C. L. Lim, B. K. DuChateau, and R. F. Schell.** 1994. Seroprotective groups of Lyme borreliosis spirochetes from North America and Europe. J. Infect. Dis. 170:115–121.
31. **Ma, J., P. A. Bulger, D. R. Davis, B. Perilli-Palmer, D. A. Bedore, C. R. Kensil, E. M. Young, C.-H. Hung, J. R. Seals, C. S. Pavia, and R. T. Coughlin.** 1994. Impact of the saponin QS-21 and aluminium hydroxide on the immunogenicity of recombinant OspA and OspB of *Borrelia burgdorferi*. Vaccine 12:925–932.
32. **Magnarelli, L. A., J. F. Anderson, H. R. Levine, and S. A. Levy.** 1990. Tick parasitism and antibodies to *Borrelia burgdorferi* in cats. J. Am. Vet. Med. Assoc. 197:63–66.
33. **Nguyen, T. P., T. T. Lam, S. W. Barthold, S. R. Telford III, R. A. Flavell, and E. Eikrig.** 1994. Partial destruction of *Borrelia burgdorferi* within ticks that engorged on OspE- or OspF-immunized mice. Infect. Immun. 62:2079–2084.
34. **Parker, J. L., and K. K. White.** 1992. Lyme borreliosis in cattle and horses: a review of the literature. Cornell Vet. 82:253–274.
35. **Peter, O., and A. G. Bretz.** 1992. Polymorphism of outer surface proteins of *Borrelia burgdorferi* as a tool for classification. Zentralbl. Bakteriol. 277:28–33.
36. **Philipp, M., Y. Lobert, R. Bohm, Jr., M. Conway, V. Dennis, P. Desmons, Y. Gu, P. Hauser, R. Lowrie, Jr., J. Piesman, and D. Roberts.** 1994. Safety and immunogenicity of recombinant OspA vaccine formulations in rhesus monkey, p. 111–114. *In* R. Cevenini, V. Sambri, and M. La Placa (ed.), Advance in Lyme borreliosis research. Proceedings of the VIth International Conference on Lyme Borreliosis. 19 to 22 June 1994, Bologna, Italy.
37. **Preac-Mursic, V., B. Wilske, E. Patsouris, S. Jauris, G. Will, E. Soutschek, S. Rainhardt, G. Lehnert, U. Klockmann, and P. Mehraein.** 1992. Active immunization with pC protein of *Borrelia burgdorferi* protects gerbils against *B. burgdorferi* infection. Infection 20:342–349.
38. **Probert, W. S., and R. B. LeFebvre.** 1994. Protection of C3H/HeN mice from challenge with *Borrelia burgdorferi* through active immunization with OspA, OspB, or OspC, but not with OspD or the 83-kilodalton antigen. Infect. Immun. 62:1920–1926.
39. **Sadziene, A., P. A. Thompson, and A. G. Barbour.** 1993. *In vitro* inhibition of *Borrelia burgdorferi* growth by antibodies. J. Infect. Dis. 167:165–172.
40. **Schaible, U. E., R. Wallich, M. D. Kramer, L. Gern, J. F. Anderson, C. Museteanu, and M. M. Simon.** 1993. Immune sera to individual *Borrelia burgdorferi* isolates or recombinant OspA thereof protect SCID mice against infection with homologous strains but only partially or not at all against those of different OspA/OspB genotype. Vaccine 11:1049–1054.
41. **Shin, J. S., Y. F. Chang, R. H. Jacobson, E. Shaw, T. L. Lauderdale, M. J. Appel, and D. H. Lein.** 1993. Cross-reactivity between *B. burgdorferi* and other spirochetes affects specificity of serotests for detection of antibodies to the Lyme disease agent in dogs. Vet. Microbiol. 36:161–174.
42. **Simon, M. M., U. E. Schaible, M. D. Kramer, C. Eckerskorn, C. Museteanu, H. K. Muller-Hermelink, and R. Wallich.** 1991. Recombinant outer surface protein A from *Borrelia burgdorferi* induces antibodies protective against spirochetal infection in mice. J. Infect. Dis. 164:123–132.
43. **Simon, M. M., U. E. Schaible, R. Wallich, and M. D. Kramer.** 1991. A mouse model for *B. burgdorferi* infection: approach to a vaccine against Lyme disease. Immunol. Today 12:11–16.
44. **Steere, A. C.** 1989. Lyme disease. N. Engl. J. Med. 321:586–596.
45. **Steere, A. C., S. E. Malawista, D. R. Snydman, R. E. Shope, W. A. Andiman, M. R. Ross, and F. M. Steele.** 1977. Lyme arthritis: an epidemic of oligoarticular arthritis in children and adults in three Connecticut communities. Arthritis Rheum. 20:7–17.
46. **Stover, C. K., G. P. Bansal, M. S. Hanson, J. E. Burlein, S. R. Palaszynski, J. F. Young, S. Koenig, D. B. Young, A. Sadziene, and A. G. Barbour.** 1993. Protective immunity elicited by recombinant Bacille Calmette-Guerin (BCG) expressing outer surface protein A (OspA) lipoprotein: a candidate Lyme disease vaccine. J. Exp. Med. 178:197–209.
47. **Straubinger, R. K., Y. F. Chang, R. H. Jacobson, and M. J. G. Appel.** Sera from vaccinated dogs, but not those from tick-infected dogs, inhibit in vitro growth of *Borrelia burgdorferi*. J. Clin. Microbiol., in press.
48. **Telford, S. R., E. Fikrig, S. W. Barthold, L. R. Brunet, A. Spielman, and R. A. Flavell.** 1993. Protection against antigenically variable *Borrelia burgdorferi* conferred by recombinant vaccines. J. Exp. Med. 178:755–758.
49. **Wilske, B., V. Preac-Mursic, U. B. Göbel, B. Graf, S. Jauris, E. Soutschek, E. Schwab, and G. Zumstein.** 1993. An OspA serotyping system for *Borrelia burgdorferi* based on reactivity with monoclonal antibodies and OspA sequence analysis. J. Clin. Microbiol. 31:340–350.